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Apoptosis is a program of cellular suicide which leads to the removal of damaged or superfluous cells without damaging overall tissue architecture. This grant concerns apoptotic induction by a 65 amino acid protein called Reaper form Drosophila melanogaster. Reaper was originally identified in a screen for critical apoptotic regulators in flies and it was later shown by our lab and others the Reaper can induce cell death upon ectopic expression in cells of both lepidopteran and vertebrate origin. In characterizing Reaper using our cell free apoptotic reconstitution system derived from Xenopus eggs, we identified a Reaper-interacting protein called Scythe that promoted cytochrome c release form the mitochondria. The goal of the proposed research has been to determine the mechanism whereby Reaper and Scythe cooperate to induce mitochondrial cytochrome c release and eventual cell death.

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Introduction

Apoptosis is a program of cellular suicide which leads to the removal of damaged or superfluous cells without damaging overall tissue architecture. This grant concerns apoptotic induction by a 65 amino acid protein called Reaper from Drosophila melanogaster. Reaper was originally identified in a screen for critical apoptotic regulators in flies and it was later shown by our lab and others that Reaper can induce cell death upon ectopic expression in cells of both lepidopteran and vertebrate origin. In characterizing Reaper using our cell free apoptotic reconstitution system derived from Xenopus eggs, we identified a Reaper-interacting protein called Scythe that promoted cytochrome c release from the mitochondria. The goal of the proposed research has been to determine the mechanism whereby Reaper and Scythe cooperate to induce mitochondrial cytochrome c release and eventual cell death.

Body of Report:

Our technical objectives were to identify the proteins responsible for inducing cytochrome c release and to clone and characterize them. As detailed in last year's report, we found that Scythe interacted directly with Hsp70 to inhibit its function and that Reaper could trigger release of Hsp70 from Scythe, thereby reactivating Hsp70 and allowing Hsp70-mediated protein folding. Therefore, our hypothesis became that Reaper/Scythe targeted folding or conformation changes of a particular protein at the mitochondria to trigger cytochrome c release. In support of this, we found that purified Scythe, Hsp70, and Reaper were sufficient to release cytochrome c from purified mitochondria. These experiments essentially satisfied **Technical objectives 1 and 2** of the original proposal.

Given the above results, it seemed that deciphering the mechanism for localization of Reaper to the mitochondria was key for understanding Reaper-induced apoptosis. Therefore, we undertook an extensive mutagenesis of Reaper and identified a region, termed the GH3 domain (a Grim homology domain, shared with the pro—apoptotic protein, Grim) that was critical for mitochondrial localization of the protein. Specifically, deletion of this region or point mutations within this region of Reaper were capable of disrupting Reaper localization to the mitochondria. Moreover, the GH3 domain was sufficient to confer mitochondrial localization on a heterologous protein, so all determinants for localization resided within this domain. While this was interesting, the truly surprising discovery was made when we examined the function of the mutant proteins in both Xenopus egg extracts and Drosophila cells. The emerging literature suggests that cytochrome c release is not important for apoptosis in Drosophila. Hence, we were perplexed as to the relevance of our findings for fly apoptosis (the organism from which Reaper was cloned). Surprisingly, we found

that our mutant Reapers unable to localize to the mitochondria were also defective in fly cell apoptosis. This suggested that mitochondria were in some way contributing to fly cell death.

In parallel work, I was examining the ability of Reaper to promote degradation of a class of apoptotic inhibitors known as the IAPs. IAPs are ubiquitin ligases, and, as we reported last year in Nature Cell Biology, Reaper can induce the auto-ubquitination and degradation of IAPs. I also found this year that IAPs can "fight back" by trying to ubiquitinate and promote degradation of Reaper. Thus, the balance of Reaper and IAPs in the cell determines which one "wins" to produce either cell survival or cell death. In analyzing the GH3 mutants described above, we were intrigued to find that Reaper GH3 mutants could no longer induce IAP degradation. This suggested that the mitochondrial localization might in some way contribute to IAP degradation. Alternatively, the GH3 domain was separately necessary for mitochondrial localization and IAP degradation. To distinguish between these possibilities, we decided to try to rectify the IAP degradation defect by restoring mitochondrial localization to the GH3-deficient Reaper protein. Towards this end, we appended a heterologous mitochondrial localization sequence onto the GH3 mutant Reaper. We were gratified to find that this not only restored Reaper localization, but also restored IAP degradation. Thus, Reaper localization to the mitochondria seems to be required to promote IAP auto-ubqiuitination. This work was published this year.

Given our results, we believe that one role of Reaper/Scythe is to promote mitochondrial changes which, in vertebrate cells leads to cytochrome c release and in flies leads to release of factors required for IAP degradation. We are currently following this up to isolate the factor(s) required to promote IAP degradation. We are also following up the experiments described in technical objective III to determine precisely how Scythe works on the mitochondria. Given the results with Hsp70, our leading hypothesis is that Scythe and Reaper cooperate to promote folding of pro-apoptotic Bcl-2 family members at the mitochondria. In support of this, we have found that Reaper/Scythe/Hsp70 can fold and activate Bax. We are currently looking at Bax knock-out cells to determine if this is, indeed, required for Reaper-induced apoptosis.

Key Research accomplishments:
Determination of a mechanism of Reaper mitochondrial localization

Determination that Scythe/Hsp70 protein folding is likely to be key for Reaper-induced mitochondrial changes

Identification of Reaper-induced IAP auto-ubiquitination

Characterization of IAP-induced Reaper ubquitination

Establishment of a linkage between mitochondrial localization of Reaper and IAP degradation.

Reportable outcomes:

The dynamics of Reaper-IAP interactions and the mutagenic analysis of Reaper function were published this year in two papers in the Journal of Biochemistry with myself as first author.

Olson, M.R., Holley, C., Yoo, S.J. Huh, J.R. , Hay, B.A. and Kornbluth, S. (2003). Reaper is regulated by IAP- mediated ubiquitination. *J. Biol. Chem*, **278***: 4028-34.*

Olson, M.R., Holley, C., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., and Kornbluth, S. (2003). A GH3- like domain in Reaper required for mitochondrial localization and induction of IAP degradation. *J. Biol. Chem,* **278**: 44758-44768

Conclusions:
The long term goal of my proposal was to understand how Reaper induced cytochrome c release from mitochondria. The work done so far on this grant has established that Reaper/Scythe/ and Hsp70 cooperate at the mitochondria to invoke cytochrome c release. Our data strongly argue that a protein in the mitochondrial preparation must be the relevant Hsp70 target. The most likely candidate is a pro-apoptotic Bcl-2 family member and experiments are under way to establish this. Clearly, cytochrome c release and other mitochondrial changes required Reaper localization to the mitochondria. We have identified the region of Reaper responsible for this anchoring and are currently trying to isolate the mitochondrial ligand responsible for Reaper docking. We have also made the fascinating finding that Reaper localization in flies is required for IAP destabilization by Reaper. This opens the way for experiments to determine what mitochondrial factors contribute to this degradation and whether release of mitochondrial contents in flies occurs by a similar mechanism as in vertebrates, proceeding via a Scythe-dependent pathway.

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A GH3-like Domain in Reaper Is Required for Mitochondrial Localization and Induction of IAP Degradation*

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Reaper is a potent pro-apoptotic protein originally identified in a screen for *Drosophila* mutants defective in apoptotic induction. Multiple functions have been ascribed to this protein, including inhibition of IAPs (inhibitors of apoptosis); induction of IAP degradation; inhibition of protein translation; and when expressed in vertebrate cells, induction of mitochondrial cytochrome *c* release. Structure/function analysis of Reaper has identified an extreme N-terminal motif that appears to be sufficient for inhibition of IAP function. We report here that this domain, although required for IAP destabilization, is not sufficient. Moreover, we have identified a small region of Reaper, similar to the GH3 domain of Grim, that is required for localization of Reaper to mitochondria, induction of IAP degradation, and potent cell killing. Although a mutant Reaper protein lacking the GH3 domain was deficient in these properties, these defects could be fully rectified by appending either the C-terminal mitochondrial targeting sequence from Bcl-x_L or a homologous region from the pro-apoptotic protein HID. Together, these data strongly suggest that IAP destabilization by Reaper in intact cells requires Reaper localization to mitochondria and that induction of IAP instability by Reaper is important for the potent induction of apoptosis in *Drosophila* cells.

Programmed cell death in the fly *Drosophila melanogaster* is regulated by a group of genes situated adjacent to one another on chromosome 3. A chromosomal deletion that removes three of these genes, *reaper*, *hid*, and *grim* (the H99 deletion), leads to the loss of developmental cell deaths as well as a loss of the majority of cell deaths resulting from cell-damaging stimuli such as X-irradiation (1). Moreover, ectopic expression of any of these individual genes leads to autonomous cell death in both cultured fly cells and cells of the intact fly (2–4). It has also been demonstrated that Reaper, Grim, and HID[1] can induce apoptosis in vertebrate cells, suggesting that these central regulators of fly cell death can engage evolutionarily conserved apoptotic pathways (5–7).

Under most circumstances, apoptotic cell death is executed by a group of aspartate-directed cysteine proteases known as caspases (reviewed in Ref. 8). In a healthy cell, caspases are inactive, allowing cell survival. However, in response to diverse apoptotic stimuli, cells can initiate signaling pathways leading to the activation of caspases and consequent proteolytic cleavage of key intracellular substrates. Acting in opposition to the caspases is a family of proteins known as the IAPs, which can bind to caspases and inhibit their enzymatic activity. In *Drosophila*, it appears that loss of IAP function is sufficient to induce apoptosis, suggesting that the *Drosophila* caspases are poised for activation, but are normally held in check by the IAPs (9).

Although *reaper*, *grim*, and *hid* (as well as a nearby pro-apoptotic gene, *sickle*) do not share overall sequence homology, careful examination of their sequences revealed an area of limited homology at their extreme N termini. Both biochemical and genetic experiments revealed that this domain, termed the IAP-binding motif (IBM), can bind to and functionally inhibit the IAPs. Specifically, these proteins can displace IAPs from caspases, thereby alleviating IAP-mediated caspase inhibition (10–12).

Aside from inhibiting IAP function, Reaper, Grim, and HID proteins have all been reported to promote proteosomal degradation of the IAP proteins (13–18). The IAP proteins examined in these experiments, including DIAP1 from *Drosophila* and XIAP, c-IAP1 (cellular inhibitor of apoptosis-1), and c-IAP2 from human cells, all have RING domains that can act as ubiquitin ligases (reviewed in Ref. 19). Because caspase-independent IAP degradation induced by Reaper, HID, and Grim depends upon an intact IAP RING domain, it has been generally concluded that these proteins can stimulate IAP auto-ubiquitination. Interestingly, the IAPs try to "retaliate" by promoting ubiquitination of Reaper, Grim, and HID, so the relative abundances of the IBM and IAP proteins reflect a balance between IAP auto-ubiquitination and ubiquitination of the IBM motif-containing IAP antagonists (20). We (15) and others (18) have also reported that Reaper and Grim can inhibit general protein translation, which may contribute to apo-

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[1] The abbreviations used are: HID, head involution-defective; IAPs, inhibitors of apoptosis; IBM, IAP-binding motif; DIAP1, *Drosophila* inhibitor of apoptosis-1; XIAP, X-linked inhibitor of apoptosis; GST, glutathione *S*-transferase; GH3, Grim helix 3; EGFP, enhanced green fluorescent protein; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-methoxy-Asp-fluoromethyl ketone; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; RFP, red fluorescent protein.

ptosis by preventing resynthesis of short-lived apoptotic inhibitors such as the IAPs.

Although IAP inhibition may be sufficient for apoptosis in fly cells, this may not be the case in vertebrates. Indeed, XIAP knockout mice do not exhibit any overt phenotypes (21). Although this could well indicate some functional redundancy among the IAPs, it may also reflect a requirement for additional events in the activation of caspases and cell death in vertebrate cells. In particular, many apoptotic stimuli in vertebrate systems promote release of cytochrome *c* from the intermembrane space of the mitochondria to the cytoplasm (reviewed in Ref. 22). Once cytoplasmic, the released cytochrome *c* binds to Apaf-1, which recruits and activates the initiator caspase, caspase-9. Also released from the mitochondria is SMAC, an IAP inhibitory protein that, in its cytoplasmically released form, carries an N-terminal IBM motif (23, 24). Like Reaper, HID, and Grim, SMAC can interfere with IAP-mediated caspase inhibition. However, unlike the fly IBM proteins, SMAC does not appear to be a potent cell killer, consistent with the hypothesis that vertebrate caspases require positive activation as well as a relief of IAP inhibition for full activation. It is currently unclear whether this reflects differences intrinsic to the IBM proteins themselves or differences in apoptotic regulation in the parent systems.

We have reported that recombinant Reaper protein can induce caspase activation in cell-free *Xenopus* egg extracts (25). In this system, mitochondria are absolutely required for caspase activation by Reaper. Indeed, Reaper can induce the release of cytochrome *c* from mitochondria in a pathway requiring a Reaper-binding protein known as Scythe (26). Although we have found that Scythe is a member of the BAG family of proteins that can modulate the chaperone activity of Hsp70/Hsc70, the precise means by which Reaper and Scythe cooperate to promote cytochrome *c* release is not yet clear (27). Although Reaper with a free N terminus can both inhibit and induce degradation of *Xenopus* IAPs, GST-Reaper protein lacking a free N terminus (and therefore unable to bind or modulate the IAPs) or Reaper bearing a deletion of the IBM motif (Reaper-(16–65)) can still induce caspase activation by triggering cytochrome *c* release. Similarly, Reaper-(16–65) has been shown to induce apoptosis when expressed in human cells (6). This same protein has also been reported to induce apoptosis in fly cells (albeit weakly), raising the issue of whether regions of Reaper lying outside of the IBM domain are important for its function (28).

Because Reaper lacking the IBM motif has some apoptotic activity in fly cells and can induce mitochondrial cytochrome *c* release in vertebrate cells, it has been speculated that Reaper might also have some mitochondrial effects in fly cells. Indeed, it has been reported that cytochrome *c* from fly cells, although not fully released in response to apoptotic induction, undergoes a conformational change, exposing previously masked epitopes (29). Moreover, there is a fly homolog of Apaf-1, known variously as DARK, HAC-1, or D-Apaf-1, that can, like Apaf-1, recruit and activate a fly caspase (DRONC) (9, 30–32). That being said, it has also been reported that RNA interference ablation of cytochrome *c* in cultured fly cells does not impair Reaper-induced apoptosis (33). In addition, the question of whether DARK is an obligatory participant in Reaper/Grim/HID-induced apoptosis is controversial.

In analyzing mutants of Grim, Claveria *et al.* (34) identified a pro-apoptotic region of Grim lying outside of the IBM motif, which they termed the GH3 domain. Grim lacking this domain was grossly defective in apoptotic induction; and intriguingly, wild-type (but not GH3 domain-deleted) Grim protein, localized to mitochondria. However, it was not clear why mitochondrial localization of Grim in the fly system might be important. These observations, coupled with the residual apoptotic induction by Reaper-(16–65), prompted us to explore the possible functions of Reaper lying within the C-terminal 50 amino acids of the protein. We report here that a GH3-like domain of Reaper is responsible for localizing Reaper to mitochondria. Surprisingly, we have discovered that a mutant Reaper protein lacking the GH3 domain is unable to induce IAP ubiquitination and destruction, whereas it maintains the ability to inhibit IAP-caspase interactions. This mutant protein is partially defective in apoptotic induction. We have found that restoration of mitochondrial localization achieved by appending the tail of either Bcl-x_L or HID to the GH3 mutant Reaper protein is sufficient to restore both cell killing and IAP destruction. These data demonstrate the importance of mitochondrial localization in cell killing by Reaper, highlight the importance of IAP destruction (as opposed to simple inhibition) for Reaper-induced apoptosis, and provide an unexpected link between mitochondrial localization and the ability of Reaper to promote IAP destruction.

EXPERIMENTAL PROCEDURES

S2 Cell Culture—Details of S2 cell culture and transfection were described previously (20), but S2 cells were maintained in *Drosophila* serum-free medium supplemented with L-glutamate (Invitrogen) and transfected with Cellfectin (Invitrogen). Generally, 12×10^6 S2 cells grown in T-25 flasks were transfected by mixing 10 μg of Reaper, Reaper mutant, or SMAC with 1 μg of EGFP/pCasper (a construct in which EGFP is driven by the constitutive ubiquitin promoter) and 48 μl Cellfectin. To monitor transfection efficiency, 20% of the transfection mixture was plated with 50 μM Z-VAD-fmk. The remaining cells were returned to the T-25 flasks. After 24 h, DNA mixtures were removed, and cells were resuspended in fresh serum-free medium (new Z-VAD-fmk was added to the control transfection following the media change). After 8 h, cells were treated with copper sulfate and analyzed after an additional 16 h by flow cytometry; at least 100,000 live cells were counted to determine percent GFP-positive cells. A minimum of four transfections per construct were analyzed per experiment; error bars represent S.D. values across all analyses, normalized for transfection efficiency. Steady-state DIAP1 levels in the presence of Reaper, Reaper mutants, and SMAC were determined by transfection as described above, but in the presence of constant 50 μM Z-VAD-fmk. 24 h after the media change, 1.5×10^6 GFP-positive S2 cells were collected by FACS, subjected to an additional 12 h of 700 μM copper sulfate treatment (in the presence of Z-VAD-fmk), and lysed in 1% Nonidet P-40. Protein concentrations were determined by the Bradford assay (Bio-Rad) and normalized. Lysates were briefly sonicated with SDS-PAGE buffer and analyzed by immunoblotting with anti-DIAP1 antibody (a gift from Dr. Bruce A. Hay). Equal protein loading was confirmed by immunoblotting with anti-tubulin antibody.

Live S2 Cell Confocal Fluorescence Microscopy—10 μg of Reaper-GFP or 15 μg of RFP-DIAP1 was transfected into *Drosophila* cells in the presence of 50 μM Z-VAD-fmk. 36 h after transfection, 700 μM $CuSO_4$ was added. Where indicated, cells were incubated with MitoTracker Red (Molecular Probes, Inc.) for 10 min and then returned to fresh medium (with copper and Z-VAD-fmk). Live cells in chambered coverglass trays (Lab-Tek) were examined by confocal fluorescence microscopy.

Xenopus Extract Preparation, DEVD Assay, DIAP1 Stability Assay, and Scythe Binding—Preparation of cleared (ultracentrifuged S extract) interphase egg extracts was carried out as described (15). These extracts were supplemented with 2 mM ATP, 5 mg/ml creatine kinase, and 20 mM phosphocreatine. DIAP1 stability assays (15) were performed as described previously.

Reaper Peptide—Reaper and Reaper deleted for the GH3 domain (ΔGH3 Reaper) generated as full-length untagged synthetic peptides were prepared as described previously (20).

Mutant Construction—Reaper mutants were constructed by overlap PCR and were subcloned into the *EcoRI/BamHI* sites of pRmHa-3 for analysis in insect cells, into the *NcoI/HindIII* sites of pGEX-KG for bacterial expression, into the *BamHI/NotI* sites of pEBB for expression in human cells, and into the *BamHI/XbaI* sites of pGALL-HIS3 (12) for expression in *Saccharomyces cerevisiae*. RFP-DIAP1 was constructed by subcloning RFP upstream of DIAP1 in pRmHa-3. For fluorescence-

based localizations, overlap PCR was used to subclone EGFP in-frame with and downstream of the *reaper* sequence. In some instances (*i.e.* with GFP-XLE and HIDC-GFP), an additional overlap PCR step was required to append the hydrophobic tail of Bcl-x_L (XLE for x_L tail at the end) or HID (HIDC for HID C-terminal tail) to the 3′-end of ΔGH3 Reaper-GFP.

Reaper-IAP Interaction Yeast Screen—This screen was performed as described previously (12), except that wild-type and mutant Reaper proteins were compared in their relative abilities to restore lethality to yeast grown on galactose.

Human Cell Culture—Details of human 293T cell culture, transfection, constructs, immunoblotting, affinity precipitation, and pulse-chase analyses were as described (20). Apoptotic induction by *reaper* mutants was assayed visually by cotransfection with GFP and scoring the number of apoptotic figures per field and by quantitative colorimetric assay for cleavage of DEVD-*p*-nitroanilide (BIOMOL Research Labs Inc.). For immunostaining, cells were transfected with *reaper*/pEBB. After 24 h, cells were incubated with MitoTracker Red and fixed on ice with 4% paraformaldehyde, followed by permeabilization with Triton X-100. Fixed and permeabilized cells were blocked in 2% bovine serum albumin and incubated with anti-Reaper antibody (diluted 1:500) labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody for visualization by immunofluorescence. Hoechst dye was used to visualize DNA.

RESULTS

Previous analyses of the Reaper protein have suggested that regions lying outside of the N-terminal IBM motif contribute to its full apoptotic activity (6, 28). Moreover, sequence alignment of the Reaper and Grim proteins suggested that Reaper might contain a domain similar to the GH3 domain found in Grim (34). We therefore examined more carefully the predicted structure of Reaper using Predict Protein from the Swiss Model server[2] and 3D-PSSM from the 3D-PSSM web server.[3] These analyses predicted a globular protein that would adopt a helical structure over much of its length (from Gln^{10} to Thr^{47}). When the amino acid sequences contained within this region of Reaper were arrayed on a helical wheel projection, it was evident that residues within the core of Reaper (amino acids 24–41) form an amphipathic helix with hydrophobic residues (Ile^{24}, Leu^{27}, Phe^{34}, Leu^{35}, Val^{38}, and Val^{39}) lying on one face of the helix and hydrophilic residues (Arg^{26}, Glu^{29}, Ser^{30}, Arg^{33}, Thr^{37}, and Glu^{41}) lying on the other (Fig. 1*A*), as had been proposed for the GH3/Trp block region of Grim (34). Using the KINEMAGE program,[4] these analyses were extended to produce a three-dimensional representation, with C-α atoms plotted to scale, assuming a perfect α-helical arrangement and spacing (Fig. 1*B*). Note the extended hydrophobic stretch of residues in Reaper between residues 32 and 42; this region is analogous to the GH3 domain of Grim and will hereafter be referred to in this study as the GH3 domain of Reaper.

The GH3 Domain Is Essential for Mitochondrial Localization of Reaper—As the GH3 domain of Grim had been previously implicated in localizing Grim to the mitochondria (34), we speculated that the GH3 domain of Reaper might also serve as a mitochondrial targeting sequence. To first determine whether Reaper localizes to mitochondria, we transfected *Drosophila* S2 cells with a construct encoding wild-type Reaper fused at its C terminus to GFP. Initial examination of the wild-type protein revealed a punctate cytoplasmic pattern that overlapped quite well with MitoTracker Red (Fig. 2*A*). Note that inclusion of Z-VAD-fmk was necessary to preserve MitoTracker Red staining in the face of Reaper expression, consistent with previous reports of the disruption of *Drosophila* mitochondria by caspases (29). MitoTracker Red staining could also be maintained in Reaper-transfected cells by prior RNA interference ablation of DARK (data not shown).

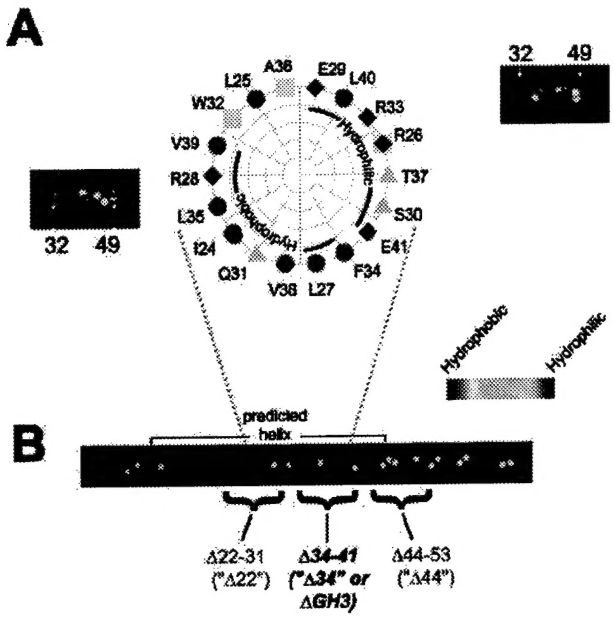

FIG. 1. **Identification of a GH3-like amphipathic helix in Reaper.** *A*, helical wheel projection of Reaper residues 24–41. Hydrophobic amino acids are represented by *red circles*, neutral amino acids by *yellow squares*, weak hydrophilic amino acids by *triangles*, and strong hydrophilic amino acids by *blue diamonds*. Residues are labeled with the single letter amino acid code and position. Note that this region forms an amphipathic helix. *Left inset*, schematic of C-α atoms of amino acids 32–49 arranged in an ideal α-helix (to scale). C-α atoms are colored as described for the helical wheel projection. Note the hydrophobic surface of this region. *Right inset*, schematic showing the opposite, hydrophilic face of amino acids 32–49. *B*, three-dimensional representation of Reaper with the extended hydrophobic stretch shown. The deletion mutants used in this study are also noted.

We confirmed that Reaper-GFP staining in the mitochondria in live S2 cells reflected the true localization of Reaper by observing that immunostained Reaper expressed in fixed human cells also co-localized with MitoTracker Red (Fig. 2*B*); GST-tagged recombinant Reaper added to *Xenopus* egg extracts could also be copurified with mitochondria (data not shown). Moreover, Reaper-GFP was as potent as untagged Reaper in apoptotic induction in S2 cells (data not shown).

To determine whether the GH3 domain is required for the mitochondrial localization of Reaper, we produced Reaper-GFP fusions bearing deletions of the GH3 domain (deleted for amino acids 34–41) or bearing similarly sized deletions flanking the GH3 domain (deletions spanning amino acids 22–31 or 44–53). As predicted by the GH3 domain homology, we found the Reaper GH3 domain (but not the flanking regions) to be essential for Reaper mitochondrial localization. However, unlike Grim, which was reported to adopt a punctate non-mitochondrial pattern when the GH3 domain was deleted (34), deletion of the Reaper GH3 domain converted Reaper to a diffusely cytoplasmic protein (Fig. 3*A*). Moreover, although wild-type Reaper appeared to be excluded from nuclei, ΔGH3 Reaper could be found throughout the cytoplasm and nucleus.

The Reaper GH3 Domain Is an Autonomous Mitochondrial Localization Sequence—Although the GH3 domain is essential for mitochondrial localization, it was not clear whether this region alone serves to target Reaper to the mitochondria. Therefore, to determine whether the Reaper GH3 domain is sufficient to confer mitochondrial localization, we appended Reaper amino acids 29–45 to GFP and examined the subcellular localization of the fusion protein in live S2 cells. As shown

[2] Available at dodo.cpmc.columbia.edu/predictprotein.
[3] Available at www.bmm.icnet.uk/.
[4] Available at kinemage.biochem.duke.edu.

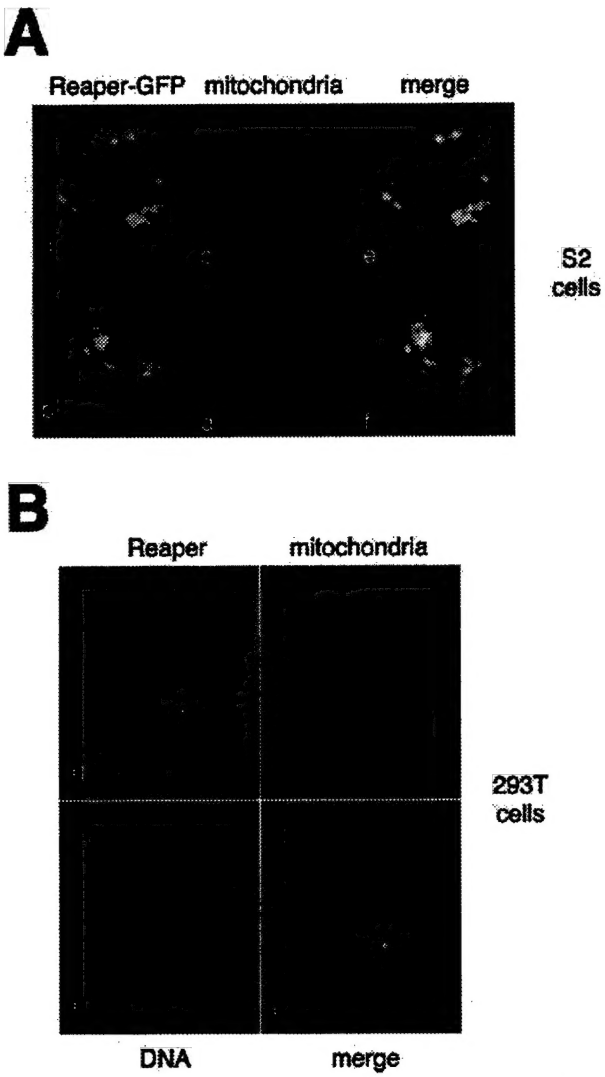

FIG. 2. **Reaper localizes to mitochondria in *Drosophila* S2 cells.** *A*, the localization of a C-terminal GFP fusion of Reaper was examined in *Drosophila* S2 cells. Two representative cells are shown with GFP fluorescence in *green* and MitoTracker Red in *red*, followed by the overlay. *B*, untagged Reaper was transfected into human 293T cells, which were fixed; permeabilized; and subjected to immunofluorescence using polyclonal anti-Reaper antibody (*panel g*), MitoTracker Red (*panel h*), and Hoechst dye (*panel i*). *Panel j* shows the human cell three-color merge.

in Fig. 3*B*, the GH3 domain fused to GFP displayed the same mitochondrial localization as wild-type Reaper. Thus, it appears that the GH3 domain is both necessary and sufficient to promote the mitochondrial association of Reaper.

Reaper GH3 Mutants Are Not Defective in Either IAP Inhibition or IAP Co-localization—Although Reaper has been shown to induce mitochondrial cytochrome *c* release in heterologous systems, it is not clear what role, if any, mitochondria might play in Reaper-induced fly cell apoptosis, particularly since the involvement of cytochrome *c* in activation of the fly apoptosome is controversial. Since ablation of DIAP1 is sufficient to cause fly cell apoptosis, and Reaper can both inhibit IAP function and induce IAP destruction, it was attractive to speculate that the defective mitochondrial localization of the GH3 mutant could in some way impact Reaper-IAP dynamics. Because short peptides encoding IBM-like motifs similar to the Reaper N terminus are sufficient to disrupt IAP-caspase interactions *in vitro*, we did not think it likely that GH3 mutants would be deficient in the direct inhibition of IAP activity. However, to address this issue, we took advantage of a genetic screen developed by Hay and co-workers (12) in which the *Drosophila* executioner caspases drICE and DCP-1 were overexpressed in *S. cerevisiae*. Caspase overexpression via the potent *GAL* promoter is lethal to yeast, and viability is rescued by *CUP1*-driven expression of DIAP1. Expression of Reaper (also driven by the *GAL* promoter) de-suppresses caspase activity, thereby killing the yeast. Using this assay, we tested wild-type *reaper*, *reaper* deleted for the GH3 domain (deletion of amino acids 34–41), and *reaper* bearing point mutations within the GH3 domain for their abilities to release drICE or DCP-1 from DIAP1. As shown in Fig. 4 (*A* and *B*), all Reaper proteins examined in this assay were able to relieve IAP-mediated caspase inhibition. Moreover, the Reaper proteins were not themselves toxic to yeast since *GAL*-driven Reaper did not impair growth of yeast on galactose *versus* dextrose when yeast were transformed with *GAL*-driven Reaper alone (Fig. 4*C*). These observations demonstrate that the GH3 mutations do not interfere with the ability of Reaper to inhibit IAP-mediated caspase inhibition. Consistent with these data, when GH3 mutants were cotransfected with DIAP1 into tissue culture cells, they co-immunoprecipitated with DIAP1 as well as wild-type Reaper, suggesting that they have the potential to bind and inhibit DIAP1 function (see Fig. 6*A*).

Although the Reaper GH3 mutants retained their intrinsic ability to inhibit IAP function, it was possible that these mutants would not co-localize with DIAP1 in an intact fly cell (as opposed to a tissue culture cell lysate or yeast cell) due to perturbation of Reaper mitochondrial targeting. We found that wild-type Reaper-GFP and RFP-DIAP1 coexpressed in S2 cells co-localized in a punctate perinuclear pattern (Fig. 5*A*) very similar to that described by Miller and co-workers (35). Intriguingly, although GH3 domain-deleted Reaper left the mitochondria, DIAP1 traveled with it, adopting a similarly diffuse cytoplasmic pattern, albeit with nuclear exclusion (Fig. 5*B*). (This is probably because unbound Reaper-GFP is small enough to diffuse through the nuclear pores, whereas RFP-DIAP1 or the DIAP1-Reaper complex is not.) Therefore, although the correct subcellular localization of the Reaper GH3 mutant is disrupted, the IAP likely remains co-localized with it in intact cells.

GH3 Mutant Reaper Protein Cannot Destabilize IAPs—Although the ΔGH3 mutant retained the ability to displace caspases from DIAP1, it remained possible that the mutant was defective in some other IAP-mediated function. We have reported previously that IAPs can ubiquitinate Reaper and that Reaper can stimulate IAP degradation (15, 20). We would predict that a Reaper mutant either particularly susceptible to IAP-mediated degradation or unable to promote IAP self-destruction would be a poor apoptotic inducer. In examining our Reaper mutants, we performed a previously described fluorescence stability assay (20) in *Drosophila* S2 cells and found that all proteins (when assayed 12 h post-transfection to ensure that endogenous DIAP1 was still present) had half-lives similar to that of wild-type Reaper (data not shown). Moreover, as we had shown previously that Reaper undergoes DIAP1-mediated ubiquitination (20), we examined the Reaper deletion mutants to see if removal of the GH3 domain prevents ubiquitin conjugation to Reaper. As expected, the GH3 domain-deleted protein was similar to wild-type Reaper in its susceptibility to DIAP1-mediated ubiquitination (Fig. 6*A*).

Acting in opposition to IAP-mediated degradation of Reaper is the Reaper-stimulated destruction of the IAPs. We sought to determine whether GH3 mutant proteins might be defective in this activity. Using an *in vitro* reconstitution assay we reported

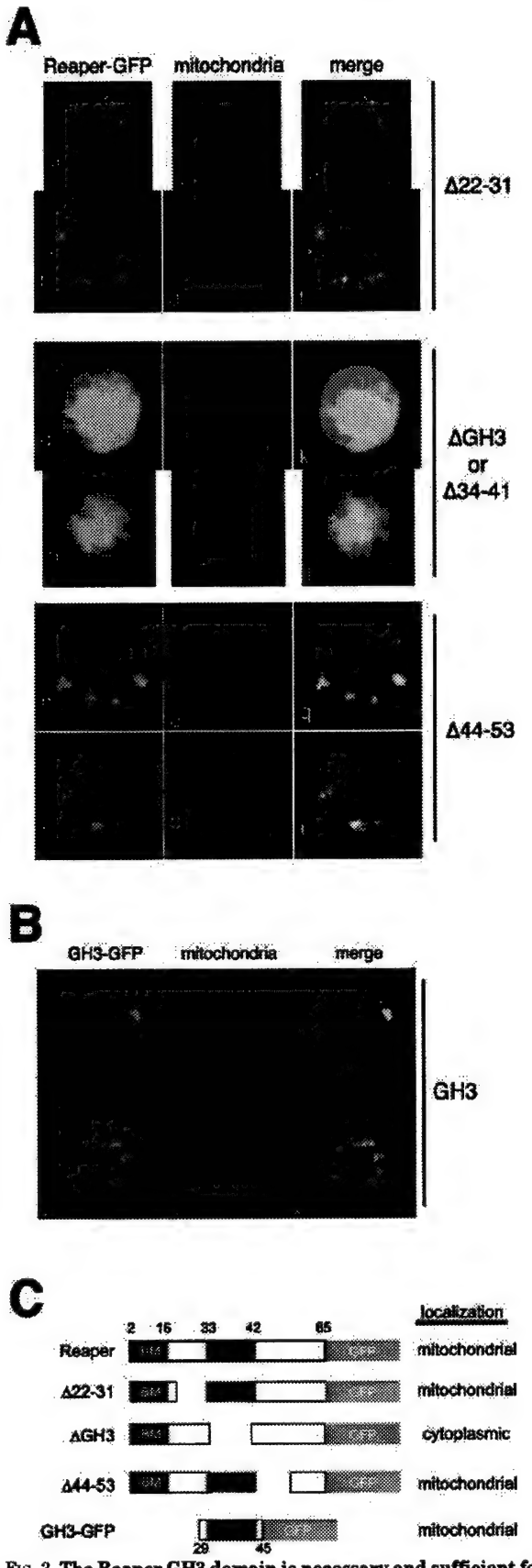

FIG. 3. **The Reaper GH3 domain is necessary and sufficient for mitochondrial localization.** *A*, Reaper-GFP mutants were transfected into *Drosophila* S2 cells that were treated as described in the legend to Fig. 2. Shown are two cells, each transfected with Δ22–31 Reaper-GFP (*panels a–f*), ΔGH3 Reaper-GFP (deletion of amino acids 34–41; *panels g–l*), and Δ44–53 Reaper-GFP (*panels m–r*). *B*, amino acids 29–45 of Reaper were fused to GFP and transfected into *Drosophila* S2 cells that were treated as described in the legend to Fig. 2. *C*, shown is a schematic of the Reaper deletions made and their respective localizations.

previously (15), a synthetic Reaper peptide missing its GH3 domain, but containing the full remainder of the Reaper coding sequence, was unable to effect DIAP1 destruction like its wild-type counterpart in *Xenopus* egg cytosol (Fig. 6*B*). Furthermore, an additional centrifugation of the cytosol used in this *in vitro* assay at 200,000 × *g* to remove contaminating membranes (Fig. 6*B*, *Double Spun*) (see "Discussion") rendered even the wild-type peptide unable to effect DIAP1 destruction.

We next examined the effect of the Reaper GH3 domain on the steady-state level of DIAP1 in *Drosophila* S2 cells. For this purpose, wild-type or ΔGH3 *reaper* driven by the metallothionein promoter was transfected into S2 cells along with GFP in the presence of Z-VAD-fmk and sorted to collect 1.5×10^6 GFP-positive cells. Wild-type and mutant Reaper proteins were then induced with copper sulfate and harvested after 24 h. Note that Z-VAD-fmk was clearly effective at inhibiting apoptosis, as all populations (including the vector control) contained equivalent percentages of GFP-positive cells, and no loss of GFP-positive cells was observed. Equivalent amounts of cell lysates were then immunoblotted with anti-DIAP1 antibody. As shown in Fig. 6*C*, DIAP1 levels were markedly decreased in cells transfected with wild-type Reaper compared with cells transfected with vector alone. In contrast, the levels of DIAP1 were comparable in ΔGH3 Reaper- and vector-transfected cells, suggesting that ΔGH3 Reaper is unable to promote IAP destruction.

To verify the steady-state DIAP1 results, we examined the ability of wild-type and ΔGH3 Reaper to induce IAP instability in human 293T cells (where it is less technically challenging to do pulse-chase analysis compared with S2 cells). As we reported previously (15), and as shown for DIAP1, Reaper was quite effective in destabilizing XIAP. Moreover, ΔGH3 Reaper was also defective in killing human cells in culture (data not shown). Accordingly, we performed pulse-chase analysis on human XIAP in the presence of Reaper mutants. These results confirmed that the ΔGH3 mutant is defective in promoting IAP destabilization (Fig. 6*D*).

GH3 Domain-deleted Reaper Is a SMAC-like Molecule—Given the shared ability of Reaper and the mammalian SMAC protein to inhibit IAP function, we wished to determine whether SMAC, which lacks any obvious GH3 domain-like sequence, would be able to destabilize IAPs. We found that expression of processed SMAC (cytoplasmically expressed with an exposed N terminus) did not induce IAP destabilization in S2 cells (Fig. 7*A*). Moreover, processed SMAC or a peptide of only its IBM domain was also unable to destabilize DIAP1 in *Xenopus* egg extracts (Fig. 7*B*). Thus, ΔGH3 Reaper is, in effect, an SMAC-like molecule: it can still bind to IAPs and compete for BIR domain binding to promote caspase release, but it has lost the ability to bring about RING domain-mediated IAP destruction.

It has been hypothesized that both IAP inhibition and destabilization contribute to full apoptotic induction by Reaper in fly cells (13–18, 36). Consistent with this suggestion, we found that the GH3 mutant Reaper protein was partially defective in apoptotic induction. Specifically, we transfected *Drosophila* S2 cells with wild-type Reaper, ΔGH3 Reaper, or control deletion mutants, along with a plasmid encoding GFP (at one-tenth the level of Reaper DNA). The percentage of GFP-positive cells was

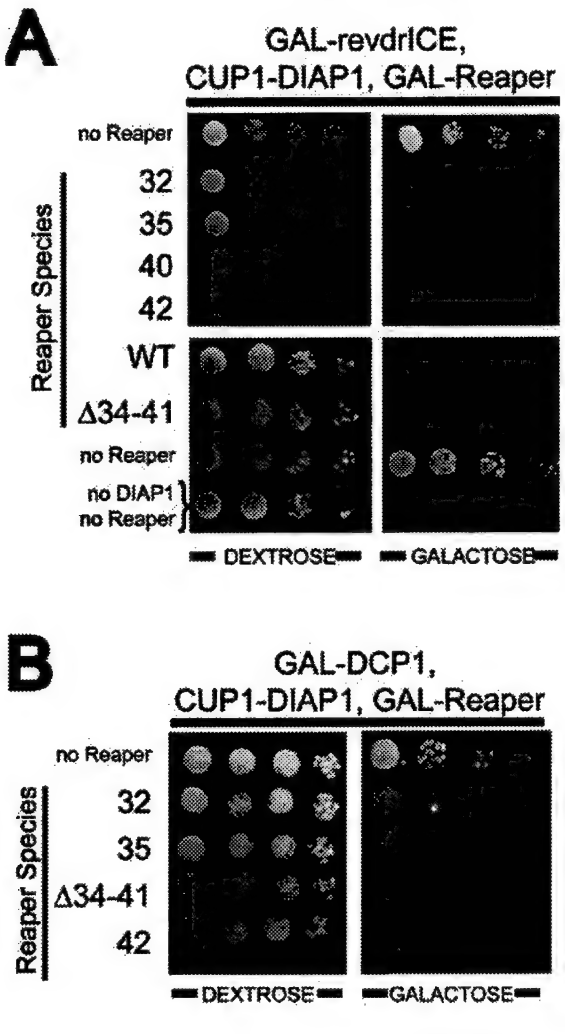

FIG. 4. **Reaper GH3 mutants retain IAP inhibitory functions.** *A*, *GAL*-driven wild-type (*WT*) or mutant *reaper* proteins were transformed into *S. cerevisiae* strain W303α together with drICE, also under *GAL* control, and DIAP1, under the control of the *CUP1* promoter. Dilution series were made of each mutant, and replicas were plated onto either dextrose-selective (*left panels*) or galactose-selective (*right panels*) medium. Note that *revdrICE* refers to the drICE in which the p10 subunit of drICE was cloned N terminal to the prodomain and the p20 subunit to obtain constitutive activity (12). For point mutants, *32* is W32I/R33L, *35* is L35Q/A36R, *40* is L40Q, and *42* is T42N/L43R. *B*, the same assay as described for *A* was carried out, but with *GAL*-DCP-1 in place of *GAL*-drICE. *C*, *S. cerevisiae* strain W303α was transformed with wild-type or mutant *reaper* genes (with vector controls in place of caspase and DIAP1).

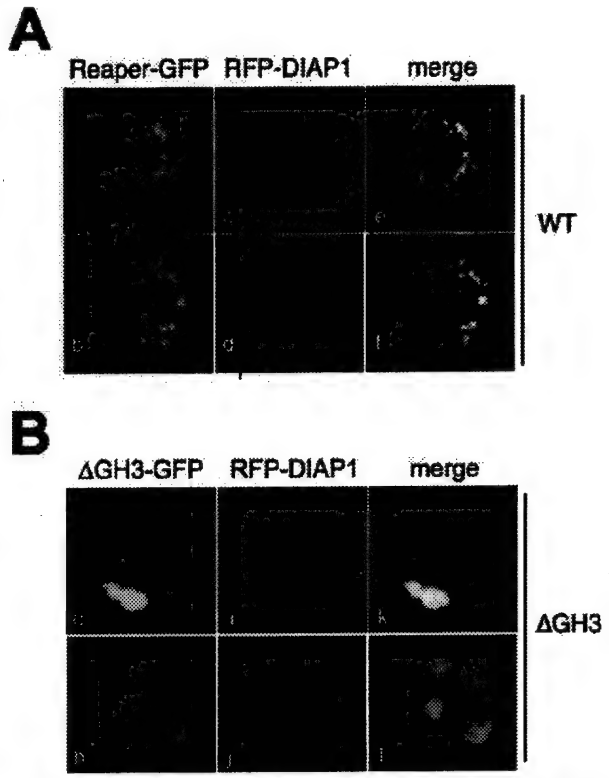

FIG. 5. **Reaper GH3 mutants co-localize with DIAP1.** Wild-type Reaper-GFP (*WT*; *A*) or ΔGH3 Reaper-GFP (*B*) was cotransfected into S2 cells with RFP-DIAP1 in the presence of Z-VAD-fink. Two-color confocal fluorescence microscopy of live S2 cells was performed as described under "Experimental Procedures." Reaper-GFP fluorescence is shown in *green*, and RFP-DIAP1 is shown in *red*. Merges are shown in *panels e*, *f*, *k*, and *l*. Note the exclusion of DIAP1 from the nucleus (*panels i* and *j*), whereas ΔGH3 Reaper is found throughout the cell (*panels g* and *h*). Wild-type Reaper and DIAP1 formed a punctate perinuclear pattern.

determined by FACS analysis and normalized for transfection efficiency. As shown in Fig. 7*C*, the GH3 domain deletion mutant was impaired in inducing S2 cell death, whereas mutants bearing similarly sized deletions on either side of the GH3 domain were as lethal as wild-type Reaper. Interestingly, SMAC was also a less potent inducer of S2 cell death than full-length Reaper, consistent with the notion that IAP inhibition alone is not as effective as the combination of inhibition and induced degradation in promoting caspase activation (data not shown). It should also be noted that changing all of the lysines in the GH3 mutant to arginine (which we have shown previously both stabilizes and enhances the biological activity of wild-type Reaper) did not rectify the apoptotic deficiencies of this mutant, consistent with the idea that increased susceptibility to IAP-mediated degradation does not underlie the impaired activity of the GH3 mutant (Fig. 7*D*). Rather, we hypothesize that the inability to promote IAP destabilization renders the GH3 mutant less active than the wild-type protein.

Restoring Mitochondrial Localization Restores ΔGH3 Reaper-mediated IAP Destabilization and Apoptosis—The experiments described above raised the issue of whether mislocalization of the ΔGH3 mutant is causally related to its inability to promote IAP degradation. Moreover, we wished to determine whether restoring mitochondrial localization would restore full apoptotic activity to the ΔGH3 mutant. To bring the ΔGH3 mutant back to the mitochondria, we took advantage of a recent study identifying the residues in mammalian Bcl-x_L that

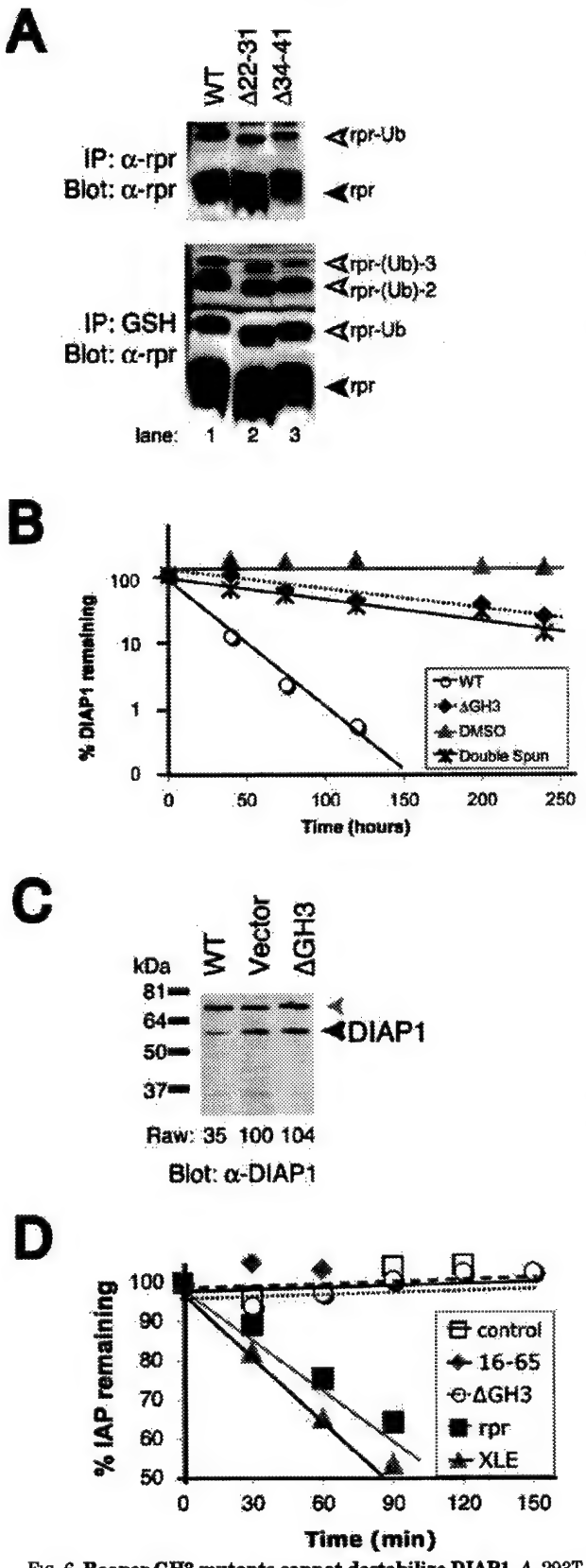

FIG. 6. **Reaper GH3 mutants cannot destabilize DIAP1.** *A*, 293T cells were cotransfected with Reaper (*rpr*) constructs and GST-DIAP1. Lysates were immunoprecipitated (*IP*) using either polyclonal anti-Reaper antiserum or glutathione beads. All Reaper proteins quantitatively precipitated with and were also ubiquitinated by GST-DIAP1 (*open arrowheads*). *Ub*, ubiquitin. *B*, the synthetic wild-type (*WT*) or ΔGH3 Reaper peptide was incubated in *Xenopus* egg cytosol, and DIAP1 stability was assayed. In addition, in one sample, the ability of the wild-type peptide to effect DIAP1 destruction was determined in cytosol that was spun for an additional 15 min at 200,000 × *g* to pellet contaminating membranes (*Double Spun*). *DMSO*, dimethyl sulfoxide. *C*, wild-type Reaper, ΔGH3 Reaper, or vector alone was transfected into S2 cells in the presence of Z-VAD-fmk. After 26 h, GFP-positive cells were isolated by FACS and induced with copper sulfate for an additional 24 h. Cell lysates were analyzed by Western blotting for endogenous DIAP1 levels. Equal loading was confirmed by immunoblotting with anti-tubulin antibody (data not shown). The parent DIAP1 species (*black arrowhead*) was quantified by densitometry and normalized to a nonspecific background band (*gray arrowhead*). *D*, Reaper or Reaper mutants (*16–65* refers to Reaper lacking amino acids 1–15; for XLE, see Fig. 8) were transfected with GST-XIAP into human 293T cells, which were then subjected to pulse-chase analysis for XIAP. At the indicated times, cells were harvested and processed by SDS-PAGE and autoradiography and then quantified.

confer its mitochondrial localization (37). Amino acids 213–233 of Bcl-x_L form the hydrophobic tail believed to anchor Bcl-x_L in the outer mitochondrial membrane. These residues were either inserted into the *reaper* sequence in place of the GH3 domain (referred to as XLM for x_L tail in the middle) or fused to the end of the ΔGH3 mutant (referred to as XLE for x_L tail at the end). Additional variants were constructed fusing GFP to these proteins, and their localizations were ascertained by confocal microscopy of live cells. As shown in Fig. 8*A*, although the XLM chimera failed to localize to the mitochondria, displaying, instead, a largely diffuse staining pattern (with some punctate non-mitochondrial concentrations), the C-terminal fusion of the Bcl-x_L residues in the XLE chimera did indeed restore mitochondrial localization of Reaper.

The killing activities of the XLE and XLM mutant Reaper proteins were determined using the FACS-based assay described above. As shown in Fig. 8*C*, the apoptotic activity of the XLE mutant was very similar to that of wild-type Reaper, whereas the XLM mutant was as defective as the parent GH3 mutant in cell killing. Indeed, we assayed this panel of mutants at a range of copper concentrations to produce varying levels of protein within the cells. At all concentrations tested, even as high as 700 μM $CuSO_4$, the ΔGH3 and XLM *reaper*-transfected cells exhibited a survival rate 3.5-fold that of the XLE or wild-type *reaper*-transfected S2 cells (Fig. 8*C*), whereas XLE and wild-type Reaper were identical. These data demonstrate that mitochondrial localization is sufficient to complement the GH3 mutant defect.

Although the Bcl-x_L tail restored apoptotic function and mitochondrial localization to the ΔGH3 mutant, it remained formally possible that the mitochondrial localization conferred upon the ΔGH3 protein a pro-apoptotic function distinct from the original IAP destabilization defect. Note, however, that all XLE-associated deaths are IAP-dependent, as deletion of the Reaper IBM motif from the XLE mutant abrogated its killing activity (Fig. 8*D*). To measure the effect of the XLE protein on IAP steady-state levels and half-lives, we transfected ΔGH3, wild-type, or XLE *reaper* into S2 cells with GFP and isolated GFP-positive cells by FACS (in the presence of Z-VAD-fmk, as described above) (Fig. 7*A*). As shown in Fig. 8*E*, the Bcl-x_L mitochondrion-anchoring tail appended to the GH3 domain-deleted protein (XLE) completely restored the ability of Reaper to promote IAP degradation. Therefore, restoring the mitochondrial targeting function is sufficient to restore IAP loss. To confirm that this loss was due to increased IAP degradation, we performed pulse-chase analysis on XIAP in Reaper-transfected 293T cells (Fig. 6*D*). Consistent with the loss of DIAP1 in S2 cells, the XLE protein markedly shortened the XIAP half-life, whereas the ΔGH3 mutant did not (Fig. 6*D*). These data imply

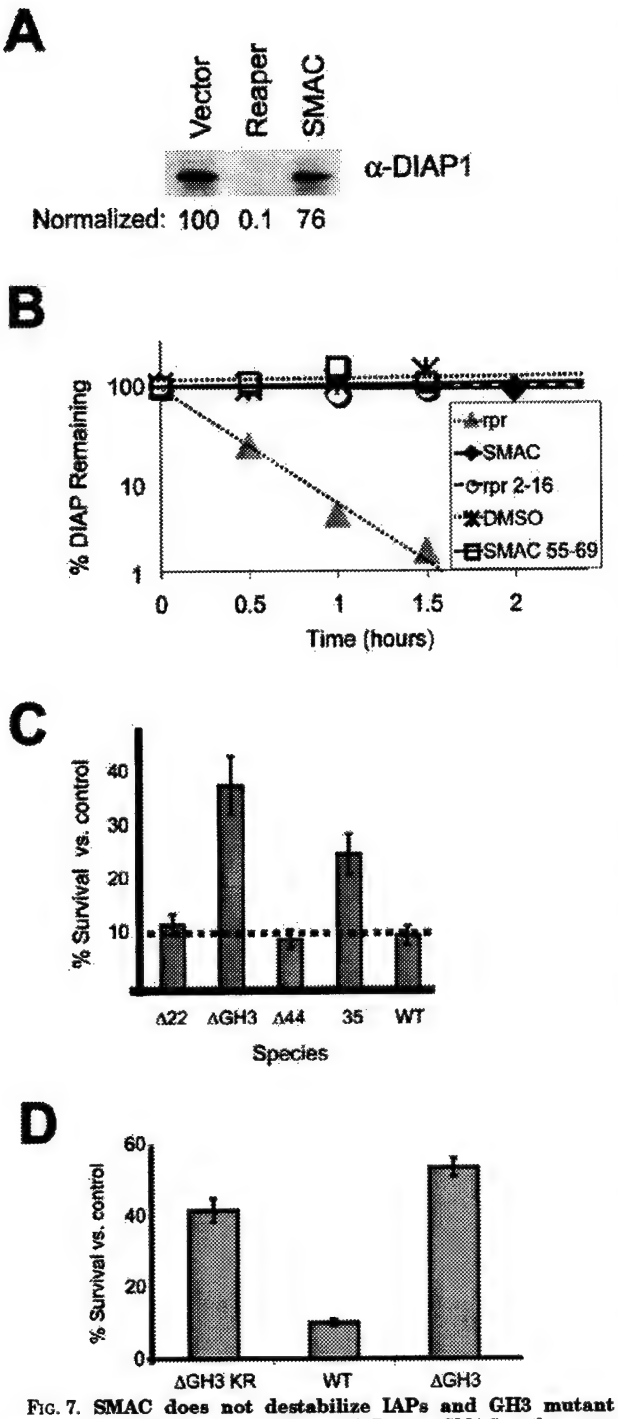

FIG. 7. **SMAC does not destabilize IAPs and GH3 mutant Reaper is a weak apoptotic inducer.** *A*, Reaper, SMAC, or the vector control was transfected into S2 cells in the presence of Z-VAD-fmk. After 36 h, GFP-positive cells were isolated by FACS and then induced with copper sulfate for an additional 24 h. Cell lysates were analyzed by Western blotting for endogenous DIAP1 levels. Equal loading was confirmed by blotting with anti-tubulin antibody (data not shown). The results from quantification by densitometry are also shown. *B*, full-length Reaper (*rpr*) peptide, the synthetic Reaper IBM domain (*rpr 2–16*), the synthetic SMAC IBM domain (*smac 55–69*), the dimethyl sulfoxide (*DMSO*) vehicle, or recombinant active Δ2–55 SMAC (*smac*) was mixed with radiolabeled DIAP1 and incubated in *Xenopus* egg cytosol (not double spun). The resulting radiolabeled protein levels were analyzed by autoradiography and quantified by densitometry. *C*, *Drosophila* S2 cells were transfected with wild-type (*WT*) or mutant Reaper constructs along with GFP as a transfection marker. Cells surviving after a 12-h induction with 7 μM copper sulfate were quantified by flow cytometry. Equal transfection efficiency was verified for all constructs by transfecting a sample of each in the presence of Z-VAD-fmk. *Error bars* represent S.D. values of four independent trials. Δ22, Δ22–31 Reaper-GFP; Δ*34*, ΔGH3 Reaper-GFP (deletion of amino acids 34–41); Δ*44*, Δ44–53 Reaper-GFP; *35*, L35Q/A36R Reaper. *D*, wild-type Reaper, ΔGH3 Reaper, or a GH3 domain deletion mutant in which all lysines were changed to arginine (Δ*GH3 KR*) was analyzed by the S2 survival assay described for *C*.

that mitochondrial targeting is important for Reaper-induced IAP degradation in intact cells.

The C-terminal Tail of HID Can Restore the Ability of ΔGH3 Reaper to Induce Apoptosis—The pro-apoptotic protein HID has also been described as a mitochondrially localized protein (5). Although HID does not contain an obvious GH3 domain, we were interested to find that the HID C-terminal tail is notably similar to the Bcl-x_L C-terminal tail (Fig. 8*F*). In fact, this observation may account for the reported ability of Bcl-x_L to displace HID from mitochondria (5), perhaps by competing for similar mitochondrial docking sites. Interestingly, loss-of-function mutants in *hid* in which this portion of HID alone has been truncated have been identified genetically (38). Unfortunately, analysis of *hid* function via overexpression of the wild-type protein is complicated by its post-translational regulation by *Drosophila* Ras (39, 40). To address the potential role of this mitochondrial targeting sequence in another manner, we wondered if we might convert the ΔGH3 Reaper protein into an effective apoptotic inducer by appending the HID tail to the end of the Reaper sequence (as the Reaper-HID chimera lacks the requisite phosphorylation sites for MAPK regulation) (Fig. 8*B*). Indeed, appending the HID tail to Reaper lacking the GH3 domain promoted its re-localization to mitochondria (Fig. 8*G*) and the induction of both apoptosis (Fig. 8*H*) and DIAP1 destruction (Fig. 8*I*). These data strongly support our conclusion that Reaper-induced DIAP1 destruction requires mitochondrial localization.

DISCUSSION

Reaper has been shown to have dual effects on IAPs, both displacing them from caspases and inducing their degradation. In previous efforts to analyze the contributions of various regions of Reaper to its biological function, it was discovered that the ability to inhibit IAP function resides in the extreme N terminus of the protein. However, this region did not appear to be sufficient for IAP destabilization, implicating additional regions of Reaper in the process. In this report, we have identified a region of Reaper, lying between amino acids 34 and 41, termed the GH3 domain of Reaper, that proved to be critical for both localizing Reaper to the mitochondria and promoting IAP destabilization. Surprisingly, restoration of mitochondrial localization by appending the tail of either Bcl-x_L or HID to a GH3 domain-deleted Reaper mutant also restored IAP instability, suggesting that mitochondrial localization may be important for this biological function of Reaper. In addition, the impaired apoptotic activity of the ΔGH3 mutant suggests that IAP destabilization, and not just IAP inhibition, is important for potent cell killing by Reaper.

IBM Motif-containing Proteins Do Not Necessarily Destabilize IAPs—As we reported previously, the mutant Reaper-(16–65) protein, which cannot bind IAPs, is also unable to induce IAP ubiquitination (15). Thus, the IBM domain is required for Reaper to stimulate IAP destruction. However, an IBM domain alone does not appear to be sufficient to induce IAP degradation, as the mammalian protein SMAC, which is unrelated to Reaper outside of the IBM domain, was unable to induce DIAP1 degradation in either fly cells or *Xenopus* egg extracts, even when expressed in its truncated, "activated" form. In

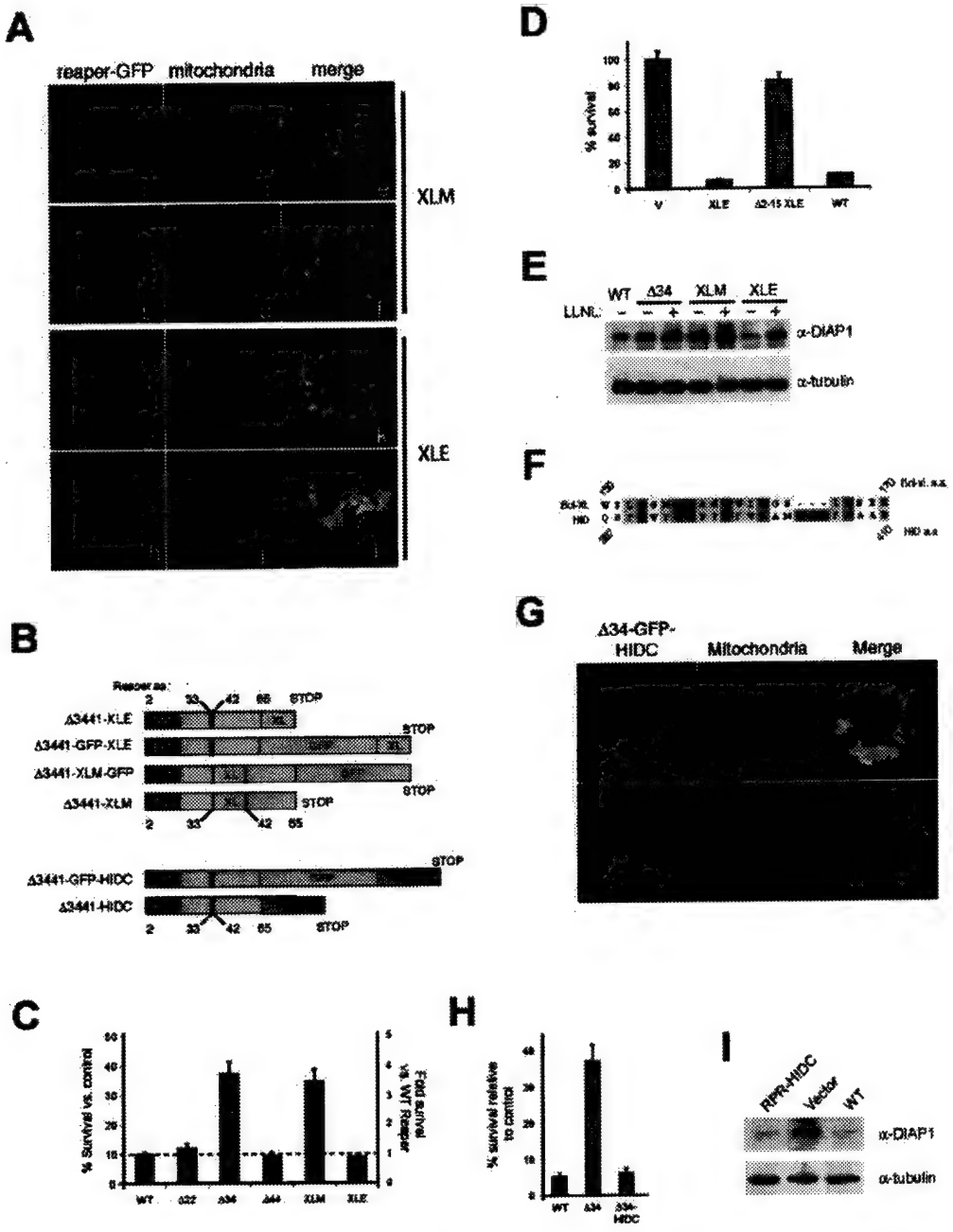

FIG. 8. **Correcting mitochondrial localization of the Reaper GH3 mutant restores apoptotic activity.** Reaper lacking the GH3 domain was fused to amino acids 213–233 of human Bcl-x_L (to make XLE), or those 21 amino acids from Bcl-x_L (minus the stop codon) were substituted for the GH3 residues of Reaper (to make XLM). In addition, the Bcl-x_L region was fused to the C terminus of ΔGH3 Reaper-GFP (to make GFP-XLE). GFP-tagged XLM was also constructed. *A*, GFP-XLE or XLM-GFP was transfected into *Drosophila* S2 cells and visualized as described in the legend to Fig. 3. *aa*, amino acids; *Δ34*, ΔGH3 Reaper (lacking amino acids 34–41); *XL*, Bcl-x_L tail. *B*, shown is a schematic of the constructs made. *C*, the *Drosophila* S2 cell survival assay was performed using all deletion mutants, expressed with 0.7 μM copper sulfate (percent survival *versus* control). In addition, a range of copper concentrations was used (from 0.7 to 700 μM) to induce varying levels of protein expression. For each mutant, at each concentration, survival (as measured by percent GFP-positive cells) was compared with survival in the presence of wild-type (*WT*) Reaper (*Fold survival vs. WT Reaper*). Note that survival plots are identical across a wide range of copper concentrations. *Error bars* represent S.D. values of results across six concentrations of copper sulfate (per construct). *Δ22*, Δ22–31 Reaper-GFP; *Δ44*, Δ44–53 Reaper-GFP; *D*, the *Drosophila* S2 survival assay was carried out using wild-type Reaper, XLE, XLE missing its IBM domain (*Δ2–15 XLE*), or vector in the presence of 7 μM copper sulfate. *E*, steady-state DIAP1 levels were determined in the presence of wild-type Reaper, ΔGH3 Reaper, XLE, or XLM (all with Z-VAD-fmk) in *Drosophila* S2 cells as described in the legend to Fig. 1*B*. In this experiment, *N*-acetyl-Leu-Leu-Nle-CHO (*LLNL*) was added (or not) to verify that destruction of DIAP1 was proteosome-dependent. *F*, shown is an alignment of amino acids 213–233 of Bcl-x_L and amino acids 387–410 of HID. Similarities are *lightly shaded*, whereas identities are *darkly shaded*. *G*, amino acids 387–410 of HID were appended to ΔGH3 Reaper-GFP (*Δ34-GFP-HIDC*), and its localization was determined in live S2 cells (with Z-VAD-fmk). *H*, the survival assay was carried out with S2 cells in the presence of wild-type Reaper, ΔGH3 Reaper, or ΔGH3 Reaper with amino acids 387–410 of HID fused to its C terminus (*Δ34-HIDC*) following induction with 7 μM copper sulfate. *I*, shown are the steady-state DIAP1 levels in the presence of wild-type Reaper, vector, or ΔGH3 Reaper-HIDC as described for *E*.

addition, IBM domain-exposed ubiquitin-SMAC (which undergoes cytosolic processing to expose its IBM domain (41)) was unable to induce XIAP instability in human 293T cells, and a peptide consisting of residues 2–16 of Reaper could not destabilize IAPs in *Xenopus* egg extracts.[5] These data are consistent with a role for other regions of Reaper in promoting IAP degradation.

The GH3 Domain Is a Mitochondrial Localization Sequence—Since deletion of the GH3 domain prevented mitochondrial localization of Reaper, we suspected that it might serve as an autonomous mitochondrial targeting sequence. This was confirmed when we found that the GH3 domain alone could target GFP to mitochondria. It is not yet clear precisely how this domain associates with mitochondria. However, based on sequence alignment, as noted by Claveria *et al.* (34), we suspect that the GH3 domains of Reaper and Grim will be determined to function similarly. It is interesting that the Bcl-x_L tail sufficed to complement the GH3 domain deletion in Reaper, allowing a restoration of apoptosis, given the lack of any overt homology between these two domains. Indeed, a careful examination of the micrographs of native Reaper and Bcl-x_L tail-tagged ΔGH3 Reaper revealed that their "mitochondrial" localization was not precisely identical, in that wild-type Reaper appeared to be diffuse over mitochondrial "spots," whereas Bcl-x_L tail-tagged ΔGH3 Reaper appeared to "ring" around the mitochondria, consistent with a Bcl-2-like outer membrane localization. Moreover, wild-type Reaper was concentrated largely at perinuclear mitochondria, whereas GFP-XLE was found across all mitochondria. Interestingly, the HID tail, which displays some homology to the Bcl-x_L tail, appeared to be more similar to the XLE protein than to wild-type Reaper, appearing to "ring" around the mitochondria as noted previously for wild-type HID by Steller and co-workers (5). These data might explain their observation that Bcl-x_L could compete HID from mitochondria (5) since HID and Bcl-x_L may share a similar, saturable, mitochondrial docking mechanism. Further biochemical experiments will be necessary to determine whether the Reaper GH3 domain confers binding to a specific mitochondrially localized protein (explaining its perinuclear concentration) or associates with the membrane bilayer via the hydrophobic face of its amphipathic helical region and is selective for perinuclear mitochondria for another, unknown reason.

Mitochondrial Localization and IAP Destabilization—Although it might be argued that the GH3 domain contributes independently to mitochondrial localization and induction of IAP degradation, the fact that both defects can be rectified by restoring mitochondrial localization speaks against this, particularly since mitochondrial re-localization by two different sequences (HID and Bcl-x_L tails) restored both apoptotic induction and IAP destruction. One plausible explanation for a mitochondrial requirement in Reaper-mediated IAP degradation is that Reaper (or Grim or HID) and the targeted IAP must co-localize at the mitochondrion with some cofactor (*e.g.* a ubiquitin carrier protein) required for degradation. This is an attractive explanation, but how then was Reaper-mediated IAP destruction successfully reconstituted in cytosolic extracts of *Xenopus* eggs (or in reticulocyte lysates)? Factors that are spatially separated in an intact cell can be randomly mixed in a cell-free lysate; and thus, it may be that mitochondrial localization is critical for IAP destruction only in the context of an intact cell. It is also plausible that mitochondrial components (or even intact mitochondria) were present as low level contaminants in the cell-free systems assayed. In this regard, it is interesting to note that recentrifugation of the "purified" *Xenopus* egg cytosol at 200,000 × *g* produced a cytosolic extract unable to support Reaper-stimulated IAP degradation (Fig. 6*B*). Additionally, at least in our hands, although DIAP1 was slightly unstable in the face of Reaper peptide in reticulocyte lysates, its half-life was extended by at least an order of magnitude (to ~4 h) compared with that in fly cells or *Xenopus* extract (~20 min). It is also possible that mitochondrial localization *in vivo* allows Reaper to achieve a high local concentration, whereas *in vitro* experiments may have employed levels of Reaper sufficiently high to bypass this requirement.

An alternative hypothesis to explain the link between mitochondrial localization and IAP degradation stems from our previously reported data demonstrating that IAPs can ubiquitinate and destroy Reaper (20). According to this hypothesis, ΔGH3 Reaper would not induce IAP destruction because the IAP would "win" the battle, destroying the ΔGH3 protein before Reaper-induced IAP auto-ubiquitination could occur. According to this scenario, the mitochondria would be a "safe zone" wherein Reaper could induce IAP ubiquitination without itself being destroyed. Arguing against this idea is the observation that mutating all of the lysines in the ΔGH3 mutant (which we have shown previously prevents IAP-mediated Reaper ubiquitination) does not restore its cell killing ability. Therefore, it is very unlikely that the primary defect in the ΔGH3 mutant or the rectification of this defect by the Bcl-x_L tail results from stabilization of the Reaper protein.

Finally, perhaps the most provocative hypothesis to explain the link between mitochondrial localization and IAP destabilization postulates that proteins localized within the mitochondria and required for IAP destruction are released from mitochondria by Reaper in a GH3 domain-dependent manner. As recent reports have suggested that multiple mitochondrial constituents are co-released with cytochrome *c* in vertebrate cells, the ability of Reaper to induce release of such factor(s) from fly mitochondria might be reflected in the ability of Reaper to induce cytochrome *c* release in heterologous systems. In this regard, it is interesting to note that we have also found ΔGH3 Reaper to be defective in inducing caspase activation in *Xenopus* egg extracts, where we have shown that apoptosis depends absolutely on mitochondrial cytochrome *c* release.[6] On the other hand, neither Reaper-(16–65) (Fig. 6*D*) nor the XLE protein in which the IBM domain was deleted (data not shown) could destabilize IAPs. These data strongly suggest that both the IBM and GH3 domains are required for Reaper to trigger IAP destabilization.

As mentioned above, it remains possible that the reconstitution of IAP degradation in cell-free systems reflects contamination of cytosolic extracts with mitochondrial constituents (which might be released by Reaper in intact cells and required for IAP degradation). In this respect, a recent report that DIAP1 can be degraded by a caspase-dependent N-end rule pathway raised the concern that such mitochondrial contaminants could allow Reaper-induced caspase activation, thereby leading to the observed IAP degradation (36). However, aside from the fact that caspase activation was never detected in these cytosolic extracts, the degradation we observed requires IAP intrinsic ubiquitin ligase activity, whereas the N-end rule pathway does not. Similarly, caspase inhibition (by Z-VAD-fmk or baculovirus p35) was utilized in all IAP destabilization experiments presented here (and previously (15)), and this did not prevent IAP destabilization in either intact human or fly cells. Finally, although SMAC in fly cells could promote some degree of caspase activation and cell death (in the absence of

[5] C. L. Holley and M. R. Olson, unpublished data.

[6] M. R. Olson and S. Kornbluth, unpublished data.

Z-VAD-fmk) (data not shown), SMAC failed to effect DIAP1 destruction, at least when caspases were inhibited.

In any case, the data presented in this study lead to the conclusion that simple inhibition of the IAP-caspase interaction is unlikely to be sufficient for inducing either IAP destabilization or apoptosis, even in fly cells. Rather, the C-terminal two-thirds of the Reaper protein must provide an additional function. The data presented here strongly suggest that this additional function depends upon the localization of Reaper to the mitochondria and is exerted, at least in part, through IAP destabilization.

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Reaper Is Regulated by IAP-mediated Ubiquitination*

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In most cases, apoptotic cell death culminates in the activation of the caspase family of cysteine proteases, leading to the orderly dismantling and elimination of the cell. The IAPs (inhibitors of apoptosis) comprise a family of proteins that oppose caspases and thus act to raise the apoptotic threshold. Disruption of IAP-mediated caspase inhibition has been shown to be an important activity for pro-apoptotic proteins in *Drosophila* (Reaper, HID, and Grim) and in mammalian cells (Smac/DIABLO and Omi/HtrA2). In addition, in the case of the fly, these proteins are able to stimulate the ubiquitination and degradation of IAPs by a mechanism involving the ubiquitin ligase activity of the IAP itself. In this report, we show that the *Drosophila* RHG proteins (Reaper, HID, and Grim) are themselves substrates for IAP-mediated ubiquitination. This ubiquitination of Reaper requires IAP ubiquitin-ligase activity and a stable interaction between Reaper and the IAP. Additionally, degradation of Reaper can be blocked by mutating its potential ubiquitination sites. Most importantly, we also show that regulation of Reaper by ubiquitination is a significant factor in determining its biological activity. These data demonstrate a novel function for IAPs and suggest that IAPs and Reaper-like proteins mutually control each other's abundance.

Apoptosis is a regulated form of cell death that can be triggered by a variety of intracellular and extracellular signals. Although apoptosis research has revealed a plethora of signaling pathways that can contribute to the decision of a cell to die, the ultimate responsibility for completing the cell death program (in most cases) resides with the cysteine proteases known as caspases (reviewed in Refs. 1–3). Caspase-mediated cleavage of cellular substrates underlies many of the ordered processes that occur as the dying cell shrinks, degrades its DNA, and packages the remains for subsequent phagocytosis. As such, regulation of caspase activation is a key control point for the apoptotic machinery.

Acting in opposition to the caspases are the IAPs (inhibitors of apoptosis). These proteins function, at least in part, by directly binding to and inhibiting active caspases (Refs. 4–6; reviewed in Refs. 7 and 8). Many of the IAPs also contain a RING finger domain, and like other RING finger proteins, these IAPs can function as ubiquitin ligases (9, 10). Ubiquitin ligases work in conjunction with ubiquitin-activating and ubiquitin-conjugating enzymes to covalently link ubiquitin to lysines present in the target protein. Sequential linkage of multiple ubiquitin moieties (polyubiquitination) then results in targeting of the ubiquitinated protein for destruction by the proteasome (reviewed in Ref. 11). Although it is well established that IAPs can inhibit caspases through physical binding, the significance of IAP ubiquitin-ligase activity for the anti-apoptotic function of IAPs is not yet fully understood. For example, IAPs have been reported to ubiquitinate and promote the degradation of caspases, which could clearly favor cell survival (9, 12). Conversely, IAPs can auto-ubiquitinate and thereby promote their own destruction, which might be expected to favor cell death (9, 10, 12–17).

In *Drosophila*, the caspase-inhibiting function of IAPs is antagonized by the pro-apoptotic proteins Reaper, HID, and Grim (the RHG[1] proteins). The genes encoding these proteins are closely linked, and their combined deletion results in a generalized failure of apoptosis during development of the *Drosophila* embryo (18). Expression of these proteins is tightly controlled at the level of transcription, and ectopic expression of the RHG proteins in either insect or vertebrate cells can initiate apoptosis (19–26). Although Reaper, HID, and Grim do not share overall homology, they do share a short region at their extreme N termini that is responsible for binding IAPs (the so-called RHG motif). The interaction between the RHG motif and the IAP has been suggested to preclude the IAP/caspase interaction, thereby alleviating caspase inhibition (27–30). The N-terminal RHG motif is also present in several vertebrate apoptotic regulators, including Smac/Diablo and Omi/HtrA2 (18, 19, 22, 28, 29, 31–34). As a consequence, these proteins can directly bind and inhibit IAPs in a manner similar to that of Reaper, HID, and Grim.

Recently, we and others have reported that Reaper, HID, and Grim can also promote apoptosis by stimulating IAP ubiquitination and degradation (12–17). Both IAP ubiquitin-ligase activity and the RHG motif are required for this particular interaction, but it is not yet clear whether other regions of the RHG protein or the IAP will also be required. Moreover, although Smac and Omi bind IAPs, there have been no reports that they also stimulate IAP degradation.

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[1] The abbreviations used are: RHG, Reaper/HID/Grim; LLnL and ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; Rpr, Reaper; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; GST, glutathione *S*-transferase; HID, head involution defective, XIAP, X-linked IAP; DIAP, *Drosophila* IAP; GFP, green fluorescent protein; FACS, fluorescence activated cell sorting.

In this report, we provide evidence that the interaction between the IAPs and the RHG proteins is a two-way street with regard to ubiquitination and proteasome-mediated degradation; that is, not only do the RHG proteins stimulate the ubiquitination and degradation of IAPs, but the IAPs also stimulate the ubiquitination and degradation of the RHG proteins. Our data demonstrate that the RHG motif is required for IAP-mediated degradation of Reaper, which suggests that a stable interaction between Reaper and the IAP is required for this form of regulation. Moreover, Reaper degradation can be blocked by inhibiting the proteasome, and when Reaper is stabilized by mutating potential ubiquitination sites, it becomes a markedly more potent inducer of apoptosis. Collectively, these data indicate that IAP regulators such as Reaper are targeted for degradation by IAP ubiquitin-ligase activity, and that this regulation is a significant factor in determining their biological activity.

EXPERIMENTAL PROCEDURES

Immunofluoresence and in Situ Hybridization—The following genetic crosses were used to generate the imaginal discs in Fig. 1: EnG4 × UAS-P35 (*panels A* and *D*), EnG4 × UAS-HID, UAS-P35 (*panels B, E,* and *G*), and EnG4 × UAS-Rpr, UAS-P35 (*panels C, F, H,* and *I*). DIAP1 and HID proteins were detected by indirect antibody fluorescence using the appropriate antibodies and standard techniques. HID and Reaper RNAs were detected by hybridization of digoxigenin-labeled probes, followed by dye staining.

Preparation of Recombinant Drosophila Proteins—DIAP1D20E was prepared as described previously (43) from GST-TEV-DIAP1D20E followed by TEV cleavage. pET23a-Reaper-GST was expressed in BL21(DE3)pLysS by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and purified using glutathione-Sepharose 4B (Amersham Biosciences) per the manufacturer's instructions. HID-His_6 and Grim-His_6 were purified as described previously (17). All of the proteins for *in vitro* ubiquitination assays were dialyzed against the buffer UD (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol) before use.

In Vitro Ubiquitination—*Drosophila* embryo extract was made as follows. 0–5-h-old embryos were collected and aged for 6 h at 25 °C. The embryos were dechorionated with 50% bleach, rinsed, suspended in equal volumes of buffer EX (20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM ATP, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 M sucrose) and homogenized. The supernatant was collected after centrifugation at 12,000 × *g*. The concentration of the extract was ~10 μg/μl. The ubiquitination assay was carried out as follows. Hid-His_6, Reaper-GST, or Grim-His_6 protein (100 ng each) was preincubated with 1 μl of embryo extract at room temperature for 10 min. Subsequently, DIAP1D20E (400 ng) and His-ubiquitin (Calbiochem; 3 μg total) were added in buffer UR (25 mM Tris, pH 7.5, 0.5 mM dithiothreitol, 2 mM ATP, 5 mM $MgCl_2$). The reaction was incubated at 37 °C for 40 min and stopped by adding SDS sample buffer. The ubiquitination of each protein was visualized after separation of proteins by SDS-PAGE, transfer to polyvinylidene difluoride, and immunoblotting with the appropriate antibody.

Generation of Reaper Antibody—Anti-Reaper serum was obtained by standard immunization techniques using New Zealand White rabbits and a synthetic Reaper C-terminal peptide conjugated to keyhole limpet hemocyanin (Research Genetics). The sequence used for peptide synthesis was CHPKTGRKSGKYRKPSQ.

Generation of ReaperKR—Reaper was cloned into pcDNA3 by standard techniques. Further work required removal of a vector *Msc*I site; thus Reaper/pc3 was digested with *Pvu*II and recircularized. The oligonucleotides (GATCCATGGCAGTGGCATTCTACATACCCGATCAGGCGACTCTGTTGCGGGAGGCGGAGCAGAGGGAGCAGCAGATTCTCCGCTTGCGGGAGTCACAGTGGAGATTCCTGG; CCAGGAATCTCCACTGTGACTCCCGCAAGCGGAGAATCTGCTGCTCCCTCTGCTCCGCCTCCCGCAACAGAGTCGCCTGATCGGGTATGTAGAATGCCACTGCCATG; CCACCGTCGTCCTGGAAACCCTGCGCCAGTACACTTCATGTCATCCGAGGACCGGAAGAAGGTCCGGCAGATATCGCAGGCCATCGCAAT; and CTAGATTGCGATGGCCTGCGATATCTGCCGGACCTTCTTCCGGTCCTCGGATGACATGAAGTGTACTGGCGCAGGGTTTCCAGGACGACGGTGG) were hybridized and cloned separately into Reaper-pcDNA3Δ*Pvu*II to generate clones A (N-terminal mutant) and B (C-terminal tetra-mutant). ReaperKR was generating by splicing the *Bam*HI/*Msc*I fragment from clone A into clone B above, and this insert was subcloned into the various vectors indicated.

In Vitro Translation—A variant of pcDNA3 was generated in which a c-Myc epitope tag was cloned downstream of the MCS *Xba*I site. The Reaper open reading frame minus its stop codon was cloned in frame with the Myc tag of pcDNA3-myc using standard techniques to generate Reaper-Myc. A variant of pSP64T, an *in vitro* SP6 expression vector with flanking 5′ and 3′ β-globin untranslated region and a polyadenosine tail was generated called pSP64BN. The *Bgl*II cloning site of pSP64T was replaced with an oligonucleotide encoding the multiple cloning site of pEBB, including unique *Bam*HI and *Not*I sites. ReaperKR-FLAG, Reaper-FLAG, Reaper, and ReaperΔ1–15 were subcloned into pSP64BN by standard techniques. To produce radioactive protein for half-life assays, Reaper-myc, Reaper, ReaperΔ1–15, Reaper-FLAG, ReaperKR-FLAG, Cdc25, and Grp94 templates were added at 20 ng μl^{-1} to rabbit reticulocyte lysate (Stratagene) containing 1 $\mu Ci\ \mu l^{-1}$ of S-35 Translabel (ICN), 1× ((−)-cysteine, (−)-methionine) amino acid mix and other components per manufacturer's protocol. Translated proteins were resolved by SDS-PAGE, soaked in 1 M salicylic acid for 1 h, dried, and exposed to Biomax MR film (Kodak). To assay protein stability, 100 $\mu g\ ml^{-1}$ cycloheximide was added to translated proteins, which were then incubated at 30 °C for an additional 30 and 60 min, boiled in SDS sample buffer, and processed as above.

Cell Culture, Transfections, Immunoblotting, Pulse-Chase Analysis, and Apoptosis Assay—All of the cell culture reagents were obtained from Invitrogen unless otherwise specified. Details of cell culture, vector constructs, immunoblotting, affinity precipitations, and pulse-chase analysis were as previously described (14), with the following exceptions. HEK 293T cells were plated at a density of 1×10^6 cells/10-cm dish for immunoblotting experiments and 200,000 cells/well in 6-well dishes for pulse-chase analysis and apoptosis assay. The cells were transfected 24 h after plating using a standard protocol of calcium phosphate and HEPES-buffered saline. 10-cm dishes were transfected with a total of 10 μg of DNA, and 6-well plates were transfected with a total of 1.6 μg of DNA/well. Where indicated, the proteasome inhibitor LLnL (ALLN, Calbiochem) was added to a final concentration of 20 μM for 45 min prior to harvesting cells. When appropriate, the cells were harvested by rinsing once with phosphate-buffered saline and lysing the cells on ice with buffer IP (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2.5 mM $MgCl_2$, and 1% Nonidet P-40, plus 1× Complete protease inhibitor (Roche Molecular)). Bead-bound material following affinity precipitation was washed three times with buffer IP prior to analysis by SDS-PAGE and immunoblotting or autoradiography. The apoptosis-inducing ability of Reaper and ReaperKR was assayed by co-transfecting pEGFP-C1 (Clontech) with the vectors indicated. After 48 h, live cells (as determined by forward and side scatter) were analyzed for GFP fluorescence by flow cytometry. Each transfection was performed in duplicate, with and without 50 μM zVAD-fmk (Biomol) to inhibit caspase activation, for a total of four transfections/construct. The percentage of live GFP+ cells for each construct in the absence of zVAD-fmk was normalized to the percentage of live GFP+ cells in the presence of zVAD-fmk such that the results shown indicate caspase-dependent loss of GFP+ cells while correcting for any differences in transfection efficiency between constructs.

SL2 Cell Culture—All of the cell culture reagents were obtained from Invitrogen unless otherwise specified. SL2 cells were obtained from the ATCC via the Duke Cell Culture Facility and were maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone). For transfection, 3×10^6 cells (at 5×10^6 cells/ml) were seeded in T-25 flasks. 24 h later, DNA was prepared for transfection by mixing 20–30 μg of appropriate constructs with 62 μl of 2 M $CaCl_2$ and 438 μl of sterile water. 500 μl of 2× HEPES buffered saline was bubbled in to each sample over 1 min. DNA mixtures were allowed to sit for 30 min at room temperature and were then added to cells for 16–24 h, after which cells were pelleted and resuspended in fresh medium.

SL2 Killing Assay—Enhanced GFP was subcloned into the *Eco*RI and *Xba*I sites of pCasper3, downstream of the ubiquitin promoter, using standard techniques. Wild type Reaper and ReaperKR were cloned into the *Eco*RI and *Bam*HI sites of pMT, downstream of the metallothionine promoter, using standard techniques. SL2 cells were co-transfected with 2 μg of GFP and 20 μg of pMT vector, Reaper, or ReaperKR. After 24 h, the cells were pelleted at 1,000 × *g* for 5 min and then resuspended in fresh medium. Following an additional 24 h, Reaper or ReaperKR was induced with 70 nM $CuSO_4$, and the cells were incubated at 25 °C for 3 days. FACS analysis was then performed. Transfection efficiency was controlled for by normalizing each transfection to its percentage of GFP (+) in the presence of zVAD-fmk. Each transformation was analyzed in triplicate (for a total of 18 samples: nine with zVAD-fmk and nine without). Forward and side scatter were used to gate viable cells, with the same gate settings used for all

samples. 100,000 gated cells/sample were counted and then analyzed for GFP fluorescence using FACS ANALYZER (BD). Cells with $>10^2$ GFP signal were taken as positive. The percentage of survival for sample A was calculated as [%GFP(+)$_A$ − zVAD-fmk]/[%GFP(+)$_A$ + zVAD-fmk]. The average percentage of GFP(+) was calculated, and the standard deviations for each sample were used to determine error.

Δ1–15 versus Wild Type Stability Assay—Full-length or Δ1–15 reaper-GFP fusions were generated by overlap PCR using the following oligonucleotides: 5′ reaper, GAAGGAGGATCCATGGCAGTGGCATTCTACATACCC; 5′ overlap, TATCGCAAGCCATCGCAAAGATCTATGGTGAGCAAGGGCGAG; 3′ overlap, CTCGCCCTTGCTCACCATAGATCTTTGCGATGGCTTGCGATA; and 3′ GFP, CCTCCGGATCCCTACTTGTACAGCTCGTCCATGCCGAG. Fusion open reading frames were subcloned into the *Bam*HI and *Eco*RI sites of pMT, downstream of the metallothionine promoter, using standard techniques. pIE3-DIAP-HA was a gift from Kristin White (Harvard/MGH). 10 μg of DIAP and 20 μg of full-length or Δ1–15 reaper-GFP were transfected into SL2 cells as described above. 24 h after resuspension in fresh medium, GFP fusions were induced with 700 nM $CuSO_4$ and placed into 50 μM zVAD-fmk to prevent cell death. After 16 h of induction, the cells were pelleted, washed with fresh medium, and resuspended in medium lacking copper but supplemented with 50 μM zVAD-fmk. The cells were immediately subjected to FACS analysis as above, with additional analyses at 16 and 24 h. Each sample was analyzed in triplicate as above. The averages were calculated for each sample, and the standard deviations were used to determine error. The percentage of Reaper remaining in the presence of DIAP1 = [+D]/[no D] where [+D] = [% GFP(+) with DIAP at time *T*]/[% GFP(+) with DIAP at time 0], and [no D] = [% GFP(+) without DIAP at time T]/[% GFP(+) without DIAP at time 0].

RESULTS

IAPs Can Ubiquitinate Reaper, HID, and Grim—Our previous work and that of others have shown that the interaction between IAPs and Reaper (and Grim and HID) lowers cellular IAP levels by stimulating ubiquitin-mediated degradation of the IAPs (12–17). In the course of these experiments involving Reaper, HID, and DIAP1, we noticed that overexpression of Reaper in *Drosophila* imaginal discs led not only to lower DIAP1 levels but also to elevated levels of HID as detected by immunofluorescence (Fig. 1, *A–F*). The reciprocal experiment examining Reaper protein levels in the presence of HID overexpression was uninformative because of the inability of our Reaper antibodies to detect Reaper *in situ*. To eliminate the possibility that Reaper expression was affecting HID transcription (Fig. 1*F*), we analyzed the amount of HID mRNA by *in situ* hybridization and found that there was no increase in the amount of HID message (Fig. 1*H*). These results implied that the regulation of Reaper and HID levels were somehow linked at a post-transcriptional step. This seemed particularly significant in light of the fact that the Reaper, HID, and Grim proteins all play a key role in developmental and radiation-induced apoptosis in *Drosophila*, but there have been no published reports on post-transcriptional mechanisms controlling the abundances of these proteins.

Although an effect on translational regulation remained a possible explanation for the elevated HID levels, this seemed unlikely because Reaper has been shown to suppress rather than enhance translation (14, 17). However, we had also noted that Reaper immunoprecipitates from cells transfected with both Reaper and a human IAP (XIAP) contained a prominent 18-kDa species recognized by anti-Reaper immunoblotting. Given the established link between the RHG proteins, IAPs, and ubiquitin-mediated degradation, we strongly suspected that this species was monoubiquitinated Reaper. Indeed, the 18-kDa band evident in the Reaper immunoblot of the anti-Reaper immunoprecipitate was also recognized by anti-ubiquitin antibody (Fig. 2*A*). Given that *Drosophila* IAP-1 (DIAP1) is the physiologically relevant IAP with regard to Reaper, we repeated these experiments with Reaper and DIAP1 with similar results (Fig. 2*A*).

We then transfected cells with Reaper and either XIAP or

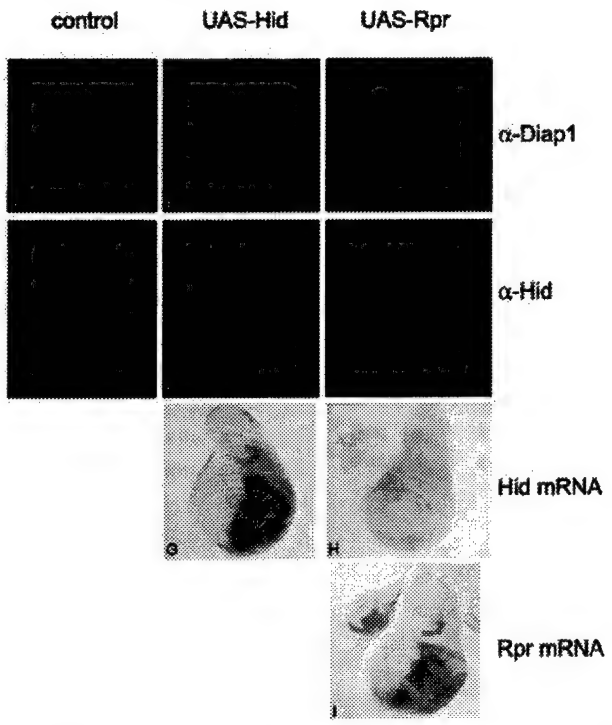

FIG. 1. **Reaper expression lowers DIAP1 levels and post-transcriptionally increases HID levels.** DIAP1 and HID were detected by immunofluorescence of imaginal discs from *Drosophila* embryos overexpressing p35 (*A* and *D*), HID + p35 (*B* and *E*), and Reaper + p35 (*C* and *F*). Expression of the caspase inhibitor p35 prevented Reaper- and HID-induced apoptosis. *in situ* hybridization was used to detect mRNA expression of HID (*G* and *H*) and Reaper (*I*).

DIAP1 and looked for Reaper-ubiquitin conjugates in the IAP immunoprecipitate. We detected multiple ubiquitinated species at 9-kDa intervals above the 9-kDa nonubiquitinated Reaper (Fig. 2*B*, *black arrowheads*). Importantly, these protein species were absent when cells were transfected with Reaper and an XIAP RING finger point mutant (H467A) that lacks ubiquitin ligase activity. Note also that only the 18- and 27-kDa species could be detected in the anti-Reaper immunoblot because the Reaper antibody was prepared against an extreme C-terminal peptide from Reaper, where four of five ubiquitin-modifiable lysines are located (see Fig. 5*A*). As such, it is likely that multiubiquitination of Reaper was interfering with antibody binding.

The presence of ubiquitinated Reaper species in the IAP co-precipitate suggested that Reaper might be a substrate of IAP ubiquitin-ligase activity. Additionally, we suspected that the RHG protein HID might also serve as a substrate for IAP-mediated ubiquitination because our initial experiment showed that HID levels increased as DIAP1 levels were lowered by Reaper (Fig. 1). To prove that Reaper and HID could in fact serve as substrates for IAP-mediated ubiquitination, we performed *in vitro* ubiquitination reactions with recombinant forms of these proteins. These results show clearly that Reaper and HID are substrates for DIAP1-stimulated ubiquitination (Fig. 2*C*). Furthermore, we found that Grim is also ubiquitinated *in vitro* (Fig. 2*C*), suggesting that all three of these *Drosophila* RHG proteins may be regulated at the level of protein stability.

Reaper Is Stabilized by Inhibiting the Proteasome—To further elucidate the machinery involved with this phenomenon, we focused on the regulation of Reaper stability and asked whether or not the proteasome was involved in the degradation

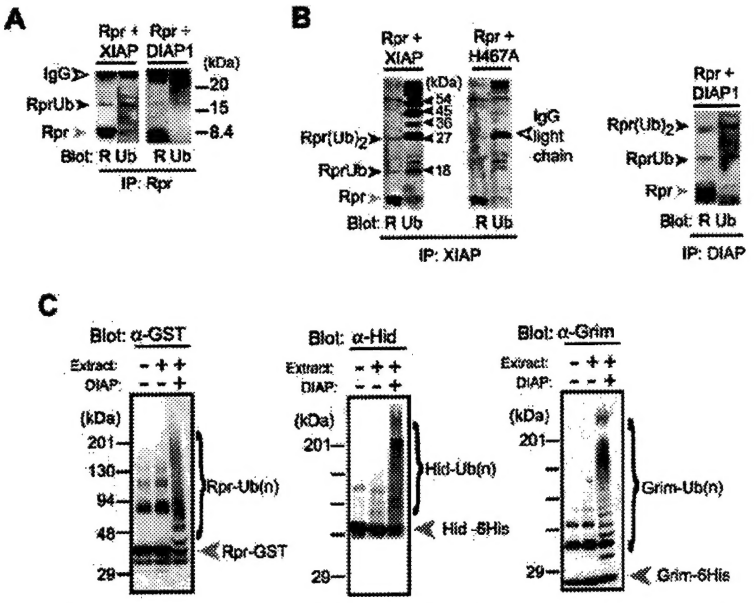

FIG. 2. **Reaper, Hid, and Grim ubiquitination.** *A*, Rpr and FLAG-tagged XIAP or Reaper and GST-tagged DIAP1 were transfected into HEK 293T cells. Immunoprecipitations were performed using anti-Reaper serum coupled to protein A (*IP: Rpr*). The precipitates were resolved by SDS-PAGE and blotted using anti-Reaper serum (*R*) or anti-ubiquitin antibody (*Ub*). The Reaper parent species is indicated (*gray arrowhead*), as is the 18-kDa monoubiquitinated species (*black arrowhead*). *B*, Rpr and FLAG-XIAP, Rpr and FLAG-H467A XIAP ligase mutant (H467A), or Rpr and GST-DIAP1 were transfected into HEK 293T cells. Affinity precipitations were performed using anti-FLAG antibody coupled to protein G (*IP: XIAP*) or glutathione-Sepharose (*IP: DIAP*). The precipitates were resolved by SDS-PAGE and blotted using anti-Reaper serum (*R*) or anti-ubiquitin antibody (*Ub*). Reaper parent species is indicated (*gray arrowhead*), as are the mono- and polyubiquitinated species (*black arrowheads*). *C*, *left panel*, recombinant Reaper-GST. *Middle panel*, Hid-His_6. *Right panel*, Grim-His_6 were mixed with *Drosophila* embryo extract for 10 min. Subsequently, recombinant DIAP1 and His-ubiquitin were added, and the mixture shifted to 37 °C for 40 min. The samples were resolved by SDS-PAGE and blotted using indicated antibodies. Note parent species (*gray arrows*) and polyubiquitinated species (*brackets*).

of ubiquitinated Reaper. When reticulocyte lysates were used to transcribe and translate Reaper, the addition of LLnL (also known as ALLN) to inhibit proteasomal degradation resulted in much higher levels of Reaper production (Fig. 3*A*). Transcription and translation of two unrelated control proteins (cdc25 and Grp94) demonstrated that the effect of LLnL was not a nonspecific increase in protein production (Fig. 3*A*). Extending these results to the more complex milieu of transfected 293T cells, we found that the addition of LLnL for 45 min significantly increased the amount of Reaper detected by immunoprecipitation and immunoblotting (Fig. 3*B*). Both experiments suggest that Reaper is targeted for proteasomal degradation.

The Reaper N Terminus Is Required for IAP-mediated Degradation—Given the ability of the RHG proteins to interact physically with IAPs, we hypothesized that this direct binding would be necessary for IAPs to promote Reaper, HID, and Grim ubiquitination and degradation. To verify this, we tested a deletion mutant of Reaper that lacked the first 15 amino acids and was therefore missing the canonical RHG IAP-binding motif (Reaper Δ1–15). In a previous report, Reaper Δ1–15 failed to co-precipitate with cellular IAP (24), and we have shown that Reaper Δ1–15 was unable to stimulate XIAP degradation (14). As expected, Reaper Δ1–15 failed to bind DIAP1 (Fig. 4*A*), whereas full-length Reaper co-precipitated with DIAP1 quite well (Fig. 2*B*). Both wild type and Reaper Δ1–15 were then transcribed and translated in rabbit reticulocyte lysates, and the results were analyzed by autoradiography. Our results showed that the Reaper Δ1–15 protein was considerably more abundant than the wild type, consistent with the mutant being more stable (Fig. 4*B*). When cycloheximide was added to the reticulocyte lysates to stop translation, the Reaper Δ1–15 protein was markedly more stable than wild type Reaper over a 60-min time course (Fig. 4*C*).

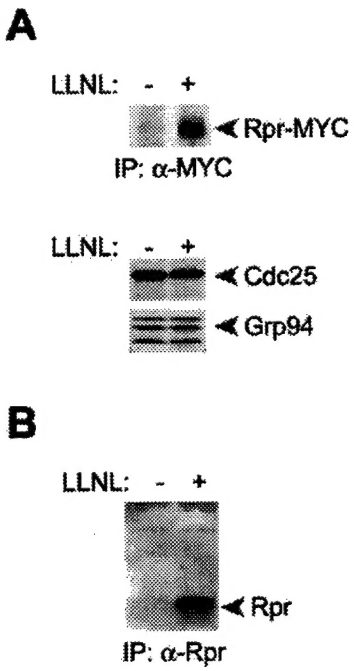

FIG. 3. **Proteasome-dependent degradation of Reaper.** *A*, Myc-tagged Reaper (Rpr-myc), Cdc25, or Grp94 were translated in the presence of 20 μM LLnL or Me_2SO carrier in rabbit reticulocyte lysate. For Reaper, equal amounts of lysate were immunoprecipitated using the 9E10 anti-Myc antibody. For controls, equal volumes of lysate were loaded for analysis. The samples were resolved by SDS-PAGE and exposed to film. *B*, untagged Reaper was transfected into HEK 293T cells that were treated for 45 min prior to harvesting with 20 μM LLnL or Me_2SO carrier. The cells were harvested and subjected to immunoprecipitation and immunoblotting using anti-Reaper serum.

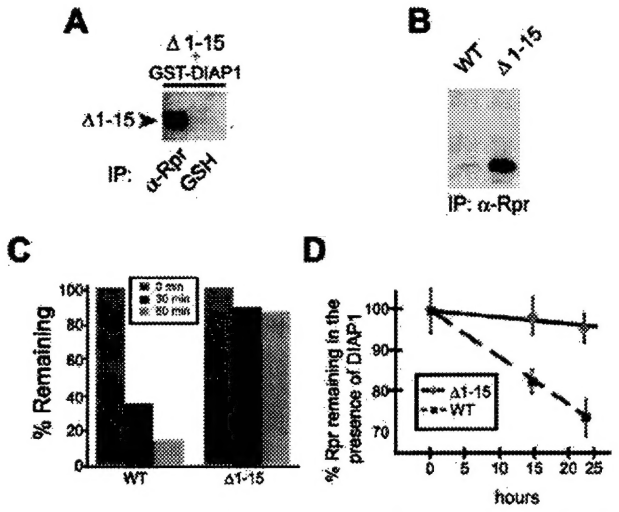

FIG. 4. **Reaper Δ1–15 is not an IAP substrate.** *A*, HEK 293T cells were co-transfected with GST-DIAP1 and Δ1–15 Reaper (Δ1–15). Co-precipitations were performed using either anti-Reaper serum (α-Rpr) or glutathione-Sepharose (*GSH*). The samples were resolved by SDS-PAGE and blotted using anti-Reaper serum. Note that Fig. 2*B* demonstrates co-immunoprecipitation of full-length Reaper and DIAP1. *B*, untagged Reaper and Reaper Δ1–15 were translated in rabbit reticulocyte lysate. Equal amounts of lysate were subject to immunoprecipitation using anti-Reaper serum. The samples were resolved by SDS-PAGE and exposed to film. *C*, reaper or Reaper Δ1–15 were produced in reticulocyte lysates, cycloheximide was added, and the proteins were incubated for a further 30 or 60 min at 30 °C. Equal volumes of reticulocyte lysate were immunoprecipitated with anti-Reaper serum and processed as above. The precipitates were resolved by SDS-PAGE and exposed to film. The results were quantified by ImageJ application (NIH). *D*, *Drosophila* SL2 cells were transfected with either copper-inducible Reaper-GFP or Δ1–15 Reaper-GFP in the presence or absence of constitutively expressed DIAP1. At time 0, copper was removed, and the cells were harvested at 0, 16, and 24 h and subjected to FACS analysis to determine the percentage of GFP-positive cells. The percentage of Reaper-GFP in the presence of DIAP1 was determined by comparing the percentage of GFP to that at time 0 in cells containing DIAP1 relative to the percentage of GFP in cells lacking DIAP1. The samples were processed and analyzed in triplicate, and the standard deviations were used to determine error. *IP*, immunoprecipitation; *WT*, wild type.

To demonstrate the biological significance of the Reaper-IAP interaction in modulating Reaper protein levels, we examined the relative stability of Reaper and Reaper Δ1–15 in *Drosophila* SL2 cells. Because SL2 cells have a low transfection efficiency, it was difficult to follow Reaper protein levels by immunoblotting or radiolabeling. Therefore, we generated Reaper-GFP and Reaper Δ1–15-GFP constructs under the control of a metallothionine promoter to perform a fluorescence-based protein stability assay. Each Reaper construct was transfected into SL2 cells, with or without DIAP1 that was driven by the constitutive baculovirus IE1 promoter. After 16 h of induction with copper, the copper containing medium was replaced with fresh (copper-free) medium, thereby inactivating the Reaper promoter. SL2 cells were then immediately analyzed for GFP fluorescence (and then analyzed again at 16 and 24 h). The results of this experiment confirmed our hypothesis that wild type Reaper was subject to DIAP1-stimulated degradation, but Reaper Δ1–15 was not (Fig. 4*D*). An identical experiment in which cycloheximide was used in place of copper-removal gave similar results (data not shown). It is worth noting that Reaper Δ1–15 retains all five of the lysines in Reaper (Fig. 5*A*), so the enhanced stability of the Reaper deletion mutant most likely stems from its inability to interact with IAPs and not from a lack of potential ubiquitin conjugation sites.

Ubiquitination-resistant Reaper Is Not Destabilized by IAPs—To fully address the biological significance of this novel mechanism for regulating Reaper levels, we wanted to ask whether or not the ubiquitination and degradation of Reaper *per se* affects its abundance and biological activity. This question precluded the use of Reaper Δ1–15 because that mutant lacks the IAP interaction domain and is therefore unable to inhibit IAP activity or stimulate IAP degradation. We therefore chose to construct an additional Reaper mutant that would interact normally with the IAPs but would itself be impervious to ubiquitination. Accordingly, we mutated all of the lysines in Reaper to arginines (ReaperKR) to inhibit ubiquitin conjugation (Fig. 5*A*). As expected, ReaperKR still interacted quite stably with DIAP1 (Fig. 5*B*). When ReaperKR was produced *in vitro* using reticulocyte lysates, we observed that much more of the mutant was made than wild type Reaper, suggesting that the lysine mutations were in fact stabilizing the protein (Fig. 5*C*). We then expressed Reaper and ReaperKR by transient transfection of 293T cells and observed that the ReaperKR produced to much higher steady state levels than wild type Reaper (Fig. 5*D*). Furthermore, although inhibition of the proteasome with LLnL increased Reaper levels, it had no effect on ReaperKR levels (Fig. 5*D*). We then performed a pulse-chase assay in 293T cells to directly examine the intrinsic half-lives of Reaper *versus* ReaperKR. As expected, the lysine mutant had a markedly increased half-life relative to wild type Reaper (Fig. 5*E*). These experiments supported our hypothesis that the ubiquitin-proteasome pathway is important for the stability of the Reaper protein and that mutation of the lysines in Reaper makes it resistant to degradation.

IAP Ligase Activity Contributes to Reaper Instability—If IAPs were in fact mediating the degradation of Reaper, we reasoned that ReaperKR should be resistant to the effects of IAP overproduction. We therefore performed pulse-chase analysis on Reaper and ReaperKR in 293T cells that had also been transfected with XIAP. The results from this assay confirmed that ReaperKR was significantly more stable than the wild type protein (Fig. 5*F*). In contrast, when the pulse-chase analysis was repeated using the XIAP H467A ubiquitin ligase mutant, wild type Reaper was stable, implying that the destabilization of Reaper was in fact specific to IAP ubiquitin-ligase activity (Fig. 5*F*).

Regulation of Reaper Stability Affects Its Ability to Induce Apoptosis—Finally, because Reaper is a potent pro-apoptotic protein, we wanted to assay the killing ability of the degradation-resistant ReaperKR with respect to wild type Reaper. We reasoned that if the regulation of Reaper levels by IAP-stimulated ubiquitination was biologically significant, then the degradation-resistant ReaperKR would be an even more potent killer than wild type Reaper. To test this hypothesis, we first compared the killing activities of Reaper and ReaperKR in transfected human cells. As shown in Fig. 6*A*, ReaperKR was a substantially better inducer of apoptosis than Reaper. Finally, to assay the biological function of Reaper in the context of its native species, we examined the relative killing activities of Reaper and ReaperKR in *Drosophila* SL2 cells. Once again, the degradation-resistant ReaperKR was a much better inducer of caspase-dependent cell death than wild type Reaper (Fig. 6*B*). Collectively, these experiments demonstrate that regulation of Reaper by IAP-mediated ubiquitination and degradation has a significant impact on the ability of Reaper to initiate apoptosis.

DISCUSSION

Our results implicate the ubiquitin-proteasome pathway in the regulation of Reaper stability and biological activity. *In vitro* ubiquitination assays coupled with overexpression and mutant studies strongly suggest that IAPs such as XIAP and DIAP1 can serve as ubiquitin ligases for Reaper, HID, and Grim. Furthermore, the regulation of Reaper stability has a

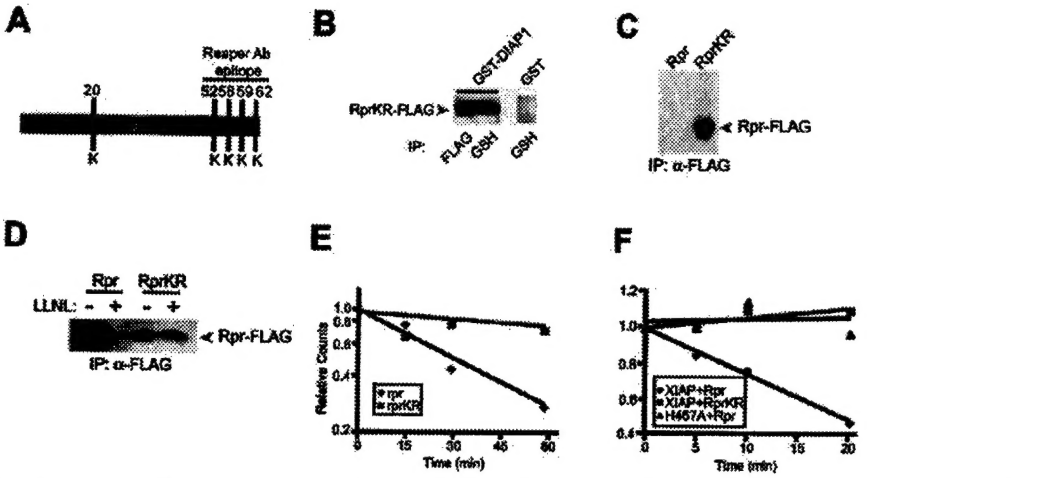

FIG. 5. **Lysine-deficient Reaper exhibits increased stability.** *A*, schematic of the Reaper protein sequence, showing the number and relative distribution of lysines. The amino acid positions are indicated. *B*, HEK 293T cells were co-transfected with GST or GST-DIAP1 and lysine-deficient Reaper (ReaperKR-FLAG). Co-precipitations were performed using either anti-FLAG antibody or glutathione-Sepharose (*GSH*). The samples were resolved by SDS-PAGE and blotted using anti-FLAG antibody. *C*, FLAG-tagged Rpr and lysine-deficient Reaper (*RprKR*) were translated in rabbit reticulocyte lysate. Equal volumes of lysate were immunoprecipitated with anti-FLAG antibody. The samples were resolved by SDS-PAGE and exposed to film. *D*, HEK 293T cells were transfected with FLAG-tagged Rpr and RprKR. 45 min prior to harvest, the cells were treated with 20 μM LLnL (or not). The cells were harvested, and the Reapers were precipitated using anti-FLAG antibody. The samples were resolved by SDS-PAGE and blotted using anti-FLAG antibody. *E*, HEK 293T cells were transfected with Rpr or RprKR. At the indicated times, the cell lysates were subjected to immunoprecipitation using anti-FLAG antibody to precipitate Rpr and RprKR. The samples were resolved by SDS-PAGE and exposed to film, and the results were quantified using the ImageJ application (NIH). *F*, HEK 293T cells were transfected with Rpr or RprKR and either GST-XIAP or GST-XIAP H467A. The samples were processed as in *E*.

significant effect on the ability of Reaper to initiate apoptosis. As such, the work reported here ascribes a new anti-apoptotic function to the IAP RING finger domain in that it promotes the degradation of RHG family members.

The findings reported here suggest that IAP proteins can ubiquitinate Reaper and its relatives and that this requires a stable interaction between the RHG protein and the IAP. We and several other groups recently reported that the interaction between Reaper, HID, and Grim and the IAPs can stimulate IAP auto-ubiquitination and degradation, thereby facilitating caspase activation and cell death (12–17). Given the ability of Reaper to stimulate IAP degradation and vice versa, it is not entirely clear how the outcome of the Reaper-IAP battle is determined. Because Reaper is also transcriptionally regulated (35–39), the balance between Reaper-mediated death and IAP-mediated survival may be partially determined by the strength of Reaper induction following a particular apoptotic stimulus. Similarly, it is likely that cells with different levels and types of IAPs will differ in their susceptibility to Reaper. Almost certainly, other modulatory factors also help to determine the outcome of the Reaper-IAP interaction. One such candidate factor is Morgue, a newly identified protein related to variant ubiquitin-conjugases that was isolated in a screen for modifiers of Reaper and Grim cell death (13, 16). This protein may assist Reaper- and Grim-mediated IAP degradation in some way, helping to shift the balance toward death when Morgue is present. Also, the ability of Reaper to suppress translation may assist in shifting the IAP-Reaper balance toward cell death.

Our data demonstrating that Reaper, HID, and Grim are all subject to IAP-stimulated ubiquitination may help to explain previous reports that have noted a cooperative apoptosis-inducing effect when more than one RHG protein is present (40, 41). Although it has been thought that this effect might be due to slightly different biological functions, the data presented here suggest that these proteins may cooperate *in vivo* by indirectly modulating each other's abundance; that is, as the RHG proteins successfully stimulate ubiquitin-mediated destruction of the IAPs, their own half-lives are extended, and they are able

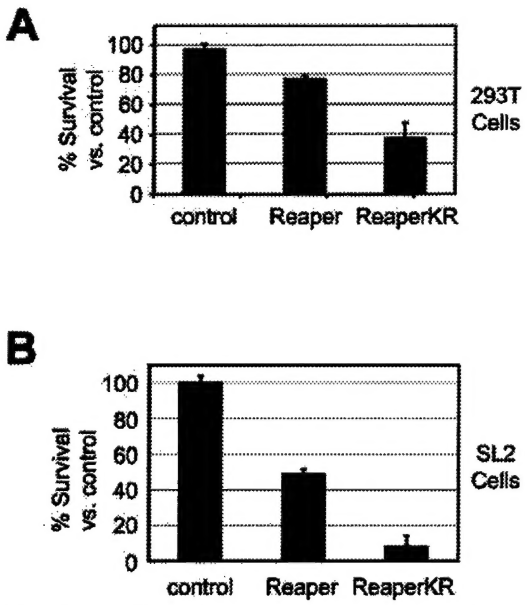

FIG. 6. **Lysine-deficient Reaper is a more potent inducer of apoptosis.** *A*, HEK 293T cells were co-transfected with GFP and vector alone (*control*), Reaper, or lysine-deficient ReaperKR in the presence or absence of the irreversible caspase inhibitor zVAD-fmk. After 48 h, the cells were harvested and subjected to FACS analysis to determine the percentage of GFP-positive cells. The percentage of survival was calculated by the percentage of GFP-positive cells without zVAD-fmk relative to the percentage of GFP-positive cells with zVAD-fmk. The samples were processed in duplicate, and the standard deviations were used to determine error. *B*, *Drosophila* SL2 cells were co-transfected with constitutively expressed GFP and metallothionine-driven Reaper, ReaperKR, or vector control. Reaper was induced 16 h after transfection (to allow for GFP expression), and the cells were harvested after a further 48 h of incubation. The cells were subjected to FACS analysis as above. The percentage of survival was calculated by dividing the percentage of GFP-positive induced cells by the percentage of GFP-positive induced cells treated with zVAD-fmk. The samples were processed in triplicate, and the standard deviations were used to determine error.

to accumulate to higher levels. This would explain the rise in HID levels following Reaper overexpression (Fig. 1).

Interestingly, the vertebrate IAP antagonist Smac is also a substrate for IAP-mediated ubiquitination, suggesting that ubiquitination of IAP-binding partners may be widespread (42). In this regard, it would be interesting to determine whether the stability of Omi is regulated by IAP proteins as well. Conversely, the weakly pro-apoptotic proteins Smac and Omi have not been reported to stimulate IAP degradation. If Smac and Omi do not, in fact, have this activity, their interaction with the IAP ubiquitin-ligase would be unidirectional, with the IAP targeting Smac and Omi for destruction, while the IAP itself remained stable. This may be the case if Smac and Omi do not engage the IAPs in precisely the same way as Reaper, HID, and Grim or if a domain in addition to the RHG motif is also required to stimulate IAP auto-ubiquitination.

Finally, the interplay that we have described between Reaper and the IAPs illustrates that the decision to undergo apoptosis (or not) is an active struggle within the cell. In this particular struggle, the outcome can be tipped one way or the other by regulating the protein stability of these antagonistic apoptotic regulators.

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